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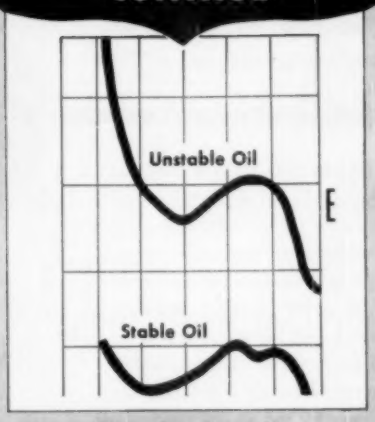

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JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

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A Study of the Interaction Between Methylcellulose and Preservatives*

By WILLIAM J. TILLMAN† and ROY KURAMOTO‡

A study has been carried out on the possible complexing tendencies of methylcellulose with preservatives used in pharmaceutical preparations. The preservatives studied were methylparaben, propylparaben, and butylparaben. Experiments were also performed on *p*-hydroxybenzoic acid, *p*-aminobenzoic acid, and ethyl aminobenzoate to determine the effect of certain functional groups on the interaction. Complexing was evidenced with all but ethyl aminobenzoate, where no apparent interaction was detected.

THE TREND towards the use of polymers in pharmacy has resulted in the inclusion of these compounds into many modern dosage forms. This in turn has presented the research pharmacist with the problem of determining the physical and chemical compatibilities between these polymers and the drugs with which they are used.

A polymer, methylcellulose, has been frequently used in formulations in which the addition of a preservative has been necessary. The possibility of an interaction between the polymer and the preservative might well prevent the preservative from performing its function and thereby make them therapeutically incompatible.

A number of investigators have studied and reported the compatibilities of methylcellulose with various compounds. Swallow and Whittet (1) reported that methylcellulose was compatible with chlorocresol 1:250, phenol 5%, and phenylmercuric nitrate. In a paper by Nakashima and Miller (2), methylcellulose was reported to show no physical or obvious chemical incompatibility with cationic medicinals. Nodzu, Forigata, and Inokawa (3) reported that there was no indication that methylcellulose contains any appreciable number of ionizing groups and that the addition of phenol and galloylgallic acid caused the coagulation of a 1% aqueous solution of methylcellulose. The addition of congo red solution to methylcellulose solutions caused gelation, while sodium phenolate, pyridine, benzoic acid, sodium benzoate, picric acid, aniline hydrochloride, methylene blue, eosin, and others have no effect. In a later paper (4) it was reported that the viscosity drop resulting from the addition of phenol or aniline to the aqueous solution of methylcellulose could be attributed to the decrease of the hydrophilic property of the molecules of methylcellulose resulting from the formation of a hydrogen bond between methylcellulose molecules and the hydroxyl and amino groups.

In a study by Swan (5) concerning the use of methylcellulose in ophthalmic solutions it was

* Received August 30, 1956, from the Columbia University College of Pharmacy, New York, N. Y.
Abstracted in part from a thesis submitted by William J. Tillman to the Graduate Faculty of Columbia University College of Pharmacy in partial fulfillment of the requirements for the degree of Master of Science.
† Present Address: School of Pharmacy, University of Wisconsin, Madison.
‡ Present Address: Ciba Pharmaceutical Products, Inc., Summit, N. J., 1955–1956 Gustavus A. Pfeiffer Memorial Research Fellow.

reported, ". . . highly surface-active compounds such as tetracaine and dibutoline sulfate . . . are adsorbed by the colloid, so that their penetration of the tissue is retarded." However, a complete explanation as to the exact mechanism of the adsorption was not offered.

Since it is apparent from all these previous investigations that very little is known about the interaction between methylcellulose and preservatives, this study was undertaken to determine whether methylcellulose does interact with these substances and to attempt to identify and explain the type of interaction.

EXPERIMENTAL

Materials.—Methylcellulose, U. S. P., 400 c. p. s. grade; methylparaben, U. S. P., m. p. 123–125°; propylparaben, U. S. P., m. p. 95–96°; butylparaben, recrystallized from alcohol and water, m. p. 66–67.5°; *p*-hydroxybenzoic acid, recrystallized from alcohol and water, m. p. 212–213°; *p*-aminobenzoic acid, U. S. P., m. p. 184–186°; ethyl aminobenzoate, U. S. P., m. p. 88–89°.

Procedures.—The method for studying the interaction of methylcellulose and the drugs was essentially the same as the solubility method outlined by Higuchi and Zuck (6).

Varying volumes of a one per cent stock solution of methylcellulose 400 c. p. s. were added to 125-ml. glass-stoppered bottles. All volumes were brought up to 50 ml., and the drug added. The bottles were then placed in a constant temperature water bath at 30°. After continuous rotation for twenty-four hours, the bottles were suspended in the water bath to allow the undissolved substances to settle and to maintain the solutions at 30° during samplings.

Aliquot samples were withdrawn and after proper dilution the solutions were assayed for their drug content on the Beckman Model DU Spectrophotometer using 1-cm. silica cells. Dilutions were as follows: methylparaben, 1:500; propylparaben, 1:125; butylparaben, 1:62.5; parahydroxybenzoic acid, 1:1000; *p*-aminobenzoic acid, 1:1000; ethyl aminobenzoate, 1:200.

The wavelength chosen for the analysis of each drug was as follows: methylparaben, 255 mμ; propylparaben, 255 mμ; butylparaben, 257 mμ; *p*-hydroxybenzoic acid, 250 mμ; *p*-aminobenzoic acid, 272 mμ; ethyl aminobenzoate, 285 mμ.

The ultraviolet absorption spectrum of a methylcellulose solution was also determined and at the concentrations and wavelengths used in this study the absorption was found to be negligible.

RESULTS AND DISCUSSION

In order to determine whether equilibrium would be attained within twenty-four hours in this study, a test experiment was set up consisting of two bottles containing distilled water and an excess of each substance studied. At the end of twenty-four and forty-eight hours, aliquot samples were removed from each bottle and, after proper dilution, assayed for their drug content. It was found that equilibrium was established at the end of twenty-four hours.

The results obtained by the described solubility procedure were most conveniently expressed as the solubility in moles per liter of the drug as a function of the concentration of the methylcellulose expressed as monomeric units per liter. The monomeric unit refers to the equivalent weight of a repeating unit of the polymer.

Methylparaben.—In the samples containing high concentrations of methylcellulose and a large excess of drug a very heavy foam formed and the viscosity of the solutions increased. This increase in viscosity was most likely due to the formation of a soluble complex (Fig. 1) which was detected in the study. An increase in viscosity of polymeric solutions in the presence of detergents was reported by Saito (7) who attributed the increase to the formation of a complex and stated that the binding was probably due to van der Waals forces exerted by alkyl chains.

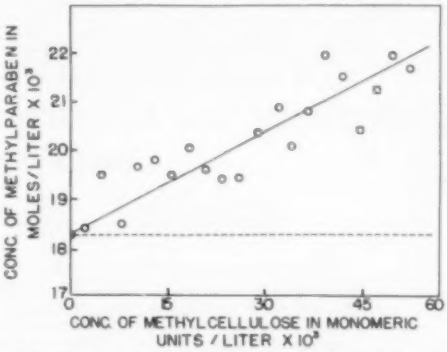

Fig. 1.—Interaction between methylcellulose and methylparaben in the presence of a large excess of methylparaben. The broken line plot represents the normal solubility of the paraben in water.

Additional studies were performed in which only a slight excess of drug was present. Here, as can be seen in Fig. 2, an insoluble complex was formed. An increase in viscosity and the formation of a heavy foam were not observed. The formation of a higher complex might have taken place in the presence of a large excess of drug. Therefore, in Fig. 1 two or

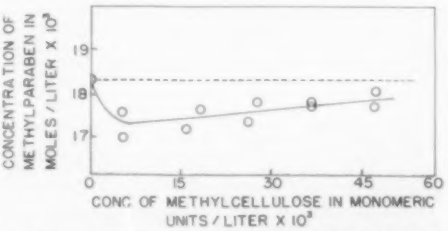

Fig. 2.—Interaction between methylcellulose and methylparaben in the presence of a slight excess of methylparaben.

more complexes with different stoichiometry may be present instead of a single complex. This phenomenon is probably explained by the study of the compatibility of methylcellulose with phenol and aniline (4). In the case of phenol, an insoluble complex was formed which caused a decrease in the viscosity of the solution. This was attributed to the formation of a hydrogen bond. In the methylparaben molecule a free hydroxyl group is available for complexing which is less acid than the phenol hydroxyl group but is still capable of forming a hydrogen bond. Evidence of the hydrogen bonding of methylparaben with hexamethylenetetramine has been reported (8).

Propylparaben.—A similar increase in viscosity and the presence of a heavy foam were observed with propylparaben. As can be seen in Fig. 3,

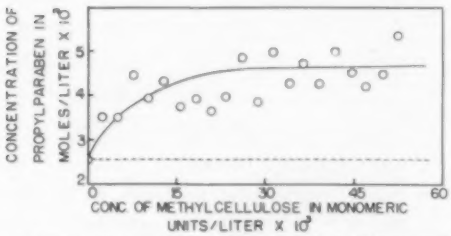

Fig. 3.—Interaction between methylcellulose and propylparaben in the presence of a large excess of propylparaben.

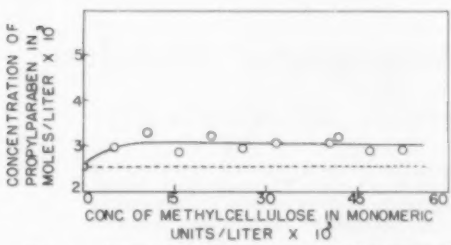

Fig. 4.—Interaction between methylcellulose and propylparaben in the presence of a light excess of propylparaben.

complex formation was detected and a soluble complex was formed. As in the case of methylparaben, studies were performed using a slight excess of drug and these results are shown in Fig. 4. The difference in the initial slopes of both curves can again be attributed to the possible formation of a higher complex in the presence of a large excess of drug rather than in the presence of a slight excess. The leveling off of the curve found in both Figs. 3 and 4 is probably due to the saturation value or maximum solubility of the complex having been reached. Any complex formed following this point would precipitate out of solution.

Butylparaben.—The butylparaben samples also increased in viscosity and formed a heavy foam. The interaction taking place (Fig. 5) would be similar to that of the methylparaben and propylparaben complex formation. It appears that the

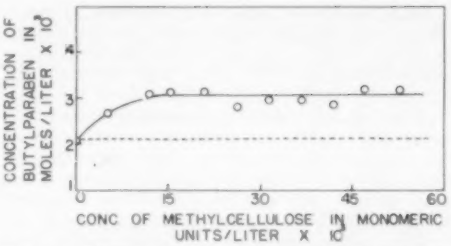

Fig. 5.—Interaction between methylcellulose and butylparaben.

presence of the butyl group which is more hydrophobic than either the propyl or methyl group would be responsible for the lower solubility of the complex. This in turn would cause the curve to level off more quickly because the saturation value of the complex would be reached sooner.

***p*-Hydroxybenzoic Acid.**—In this study a decrease in viscosity and the presence of a heavy white fibrous precipitate were noted. There appeared to be a definite decrease in the concentration of *p*-hydroxybenzoic acid in solution with increasing concentration of methylcellulose. This decrease was probably due to the formation of an insoluble complex.

The interaction between the polymer and drug was probably due to hydrogen bonding similar to that reported by Inokawa (4) in a study of the interaction of phenol and methylcellulose. It was also reported by Nodzu, *et al.* (3), that the addition of a 10% aqueous solution of phenol to methylcellulose solutions caused coagulation. This coagulation would also be expected with the *p*-hydroxybenzoic acid because of the presence of a hydroxyl group and a *para*-carboxyl group which are both capable of forming hydrogen bonds.

***p*-Aminobenzoic Acid.**—A decrease in viscosity was also observed in these studies. As shown in Fig. 6, a definite decrease in solubility was obtained. This may also be explained, as in the case of *p*-hydroxybenzoic acid, as the formation of an insoluble complex. This interaction can be attributed to hydrogen bonding due to the presence of both a carboxyl and amino group on the molecule. The increase in slope seen at the higher concentrations might be explained as the formation of a more soluble higher complex.

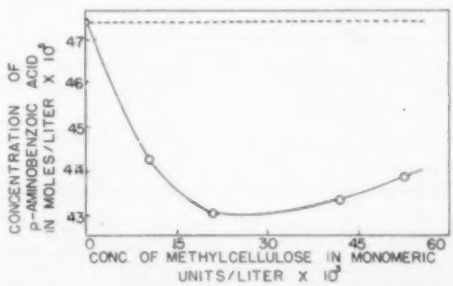

Fig. 6.—Interaction between methylcellulose and *p*-aminobenzoic acid.

Ethyl Aminobenzoate.—No evidence of complexing was detected in the studies of ethyl aminobenzoate. The viscosity of the methylcellulose solutions did not appear to change nor was there any gross physical change evident.

It was thought that there would be some interaction due to either the hydrogen bonding power of the amino group, which is less than that of the hydroxyl group or a van der Waals attraction of the alkyl group. Although interaction was expected, no apparent complexing was detected.

The results obtained in the solubility studies of all the compounds and the considerations offered coincide with those reported by Higuchi and Kuramoto (9) who stated that, "The lesser complexing tendency of the *para*-amino compound is probably due to the weaker electrophilic nature of amino hydrogen as contrasted to hydroxyl hydrogen, resulting in a weaker hydrogen bond formation." It was also stated that carboxyl groups, phenolic groups, and amino groups can all lead to definite association.

In addition to the hydrogen bonding between the molecules, a van der Waals attraction may exist. The van der Waals forces would be exerted by the alkyl chains as found in the parabens.

SUMMARY AND CONCLUSIONS

1. From the experimental data presented, it is evident that methylcellulose forms complexes in solution with certain pharmaceuticals.

2. Definite evidence of complexing between methylcellulose and methylparaben, propylparaben, butylparaben, *p*-aminobenzoic acid, and *p*-hydroxybenzoic acid was demonstrated. No apparent interaction was detected with ethyl aminobenzoate.

3. The degree of interaction between the methylcellulose and those compounds that did interact is roughly in the following decreasing order: *p*-hydroxybenzoic acid and *p*-aminobenzoic acid, methylparaben, propylparaben, and butylparaben.

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Assay of Menadione—A Polarographic Procedure*

By JAN C. JONGKIND, EDMUND BUZZA,† and SERECK H. FOX

A polarographic procedure has been developed for the determination of menadione in complex mixtures of pharmaceutical and vitamin types. The method is simple, rapid, and precise. It has been used routinely for the assay of menadione in a variety of products, some containing as many as thirty ingredients, such as oils, vitamins, antibiotics, etc.

THE INCREASED USE of menadione in multivitamin preparations in the last few years has necessitated the development of an assay for menadione which may be used routinely in the control laboratory. The paper presented here is concerned with a polarographic assay for menadione in nonaqueous solution. This assay has been applied to mixtures containing as many as thirty components.

No investigation of the theoretical aspects of the reduction of 2-methyl-1,4-naphthoquinone at the dropping mercury electrode was attempted. Efforts were confined to the establishment of the proportionality of the diffusion current with the concentration of menadione and to the estimation of the overall precision of the method, including both extraction and polarographic determination.

Onrust and Wostmann (1) determined menadione in fodder using a specially designed cell and a mixture of petroleum ether and isopropanol as solvent. They employed methylene blue as a suppressor of the maximum. Hilton (2) studied the polarographic behavior of a number of quinones, including menadione, in an aqueous bicarbonate medium. Lewis and Quackenbush (3) used a mixture of benzene and methanol, made 0.2 *M* in lithium chloride for the study of peroxides in fats and oils. The same electrolysis

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† Present address: Fisher Scientific Company, Pittsburgh, Pa.

medium was adapted to the assay reported here since menadione as well as the oils, fats, and waxes present in many of the mixtures under study are soluble in it. The polarogram of menadione in this medium shows one well-defined wave, with a half-wave potential of about −0.4 volt. Since a slight maximum occurs when pure menadione is reduced at the dropping mercury electrode, we added methylene blue when the proportionality of the diffusion current with the concentration of menadione was established, as shown in Fig. 1.

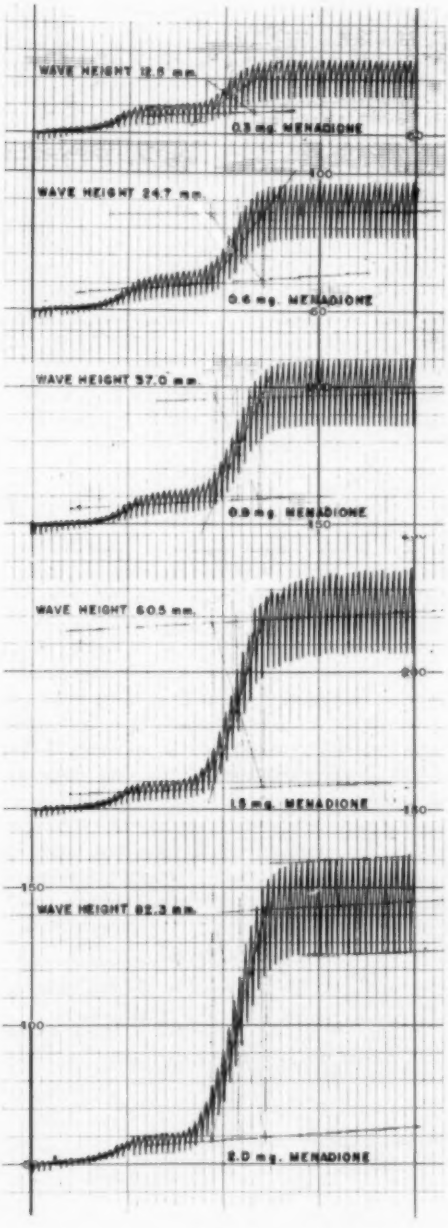

Figure 1.

EXPERIMENTAL

The polarograms shown in Fig. 1 were made using a Sargent XXI polarograph and a simple electrolysis cell with the mercury pool serving as the positive electrode. The temperature during electrolysis was held in all cases at 23°. Solutions of menadione (U. S. P. Standard) of different concentrations in benzene were made and 10-ml. aliquots transferred to the electrolysis vessel. Next, exactly 10 ml. of a 0.2 *M* solution of lithium chloride in anhydrous methanol, which also contained 0.005% of methylene blue, was added. After addition of 0.1 ml. of ammonia, nitrogen was bubbled through the solution for eight minutes to remove dissolved oxygen. In order to compensate for evaporation losses in the cell, the nitrogen was bubbled through a layer of solution of the same composition and height as the solution in the cell before being bubbled through the assay solution. The solutions were now polarographed between 0 and −1 volt, the diffusion currents measured, and the results plotted, as shown in Fig. 2.

From Fig. 2 it may be seen that there exists direct proportionality between the concentration of menadione and the diffusion current in the range studied, i. e., 0.01 to 0.1 mg. per ml.

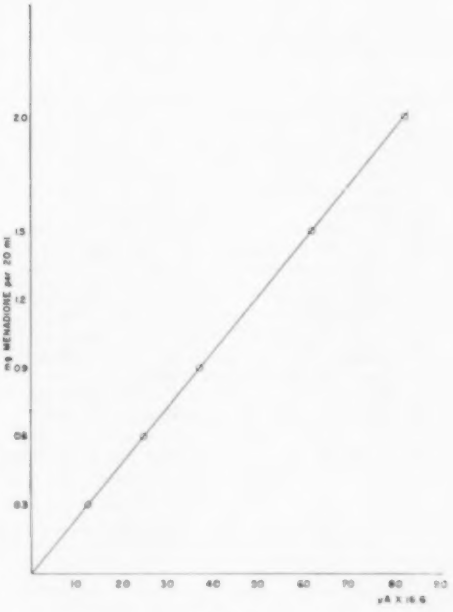

Figure 2.

Reagents.—Benzene (reagent grade), 0.2 *M* lithium chloride in anhydrous methanol, ammonia (reagent grade), cerium nitrate solution (made by dissolving 26 Gm. of $(NH_4)_2Ce(NO_3)_6$ in 1 liter of 1 *N* H_2SO_4), standard solution of menadione containing exactly 1 mg. of menadione per milliliter in a mixture of benzene and methanol 1:1 (v/v).

Procedure.—An accurately weighed sample was transferred to a 250-ml. centrifuge bottle and 25 ml. of the ceric nitrate solution added (by graduate); exactly 20 ml. benzene was then added, the bottle stoppered and placed on a mechanical shaker for 20 minutes. The mixture was then centrifuged and an aliquot of the benzene layer, containing approximately 0.5 mg. of menadione transferred to the electrolysis vessel. Enough benzene was added to the electrolysis vessel to make the volume in the cell exactly 10 ml. Ten milliliters of the lithium chloride solution (by pipet) and 0.1 ml. of ammonia were then added and nitrogen bubbled through the mixture for eight minutes, and the mixture polarographed. Exactly 1 ml. of the standard solution was then added, nitrogen again bubbled through for eight minutes, and the mixture polarographed again. The diffusion currents of both polarograms were measured and the original concentration of menadione calculated by difference.

Application of Procedure to Pharmaceutical Mixtures.—The assay procedure for menadione powder as given in the U. S. P. XV (4) does not distinguish between the quinone and hydroxyquinone forms of the compound, thus implying the equivalence of both forms in therapeutic effect. Similarly in the work reported here the two forms were not distinguished, the hydroxy form being oxidized to the quinone form before polarography.

The validity of the method was established by assay of a mixture so constructed that it represented most of the problems encountered in the assay of menadione in the field of interest. The compounded mixture was thoroughly milled to insure homogeneity. Mixture formula:

Menadione	2 mg.
Thiamine mononitrate	12.5 mg.
Riboflavin	10.7 mg.
Pyridoxine hydrochloride	0.5 mg.
Vitamin B_{12} trituration	20 mg.
Calcium pantothenate	12.5 mg.
Folic acid	0.1 mg.
Ascorbic acid	150 mg.
Nicotinamide	100 mg.
Dicalcium phosphate	365 mg.
Ferrous sulfate	51 mg.
Potassium sulfate	12 mg.
Copper sulfate	3.2 mg.
Magnesium sulfate	44 mg.
Zinc sulfate	4.2 mg.
d-Alpha tocopheryl acetate concentrate 25%	14.7 mg.
Diluent mixture of fats and oils	496.8 mg.

This "artificially prepared" mixture was assayed for menadione, using the procedure outlined above. A total of twenty determinations was made and the results tabulated, as shown in Table I.

TABLE I

Sample Weight	Diffusion Current of Sample id × 25 uA.	Diffusion Current of Sample and Standard id x 25 uA.	Results mg. K/ 1.375 Gm.
1.0410	19.5	70.0	1.932
1.0853	20.5	72.0	1.885
1.2375	23.0	73.0	1.905
1.3099	25.0	75.5	1.934
1.3680	25.5	75.5	1.906
1.0971	21.0	72.0	1.927
1.1722	23.0	73.0	2.012
1.3189	25.0	78.0	1.831
1.4018	27.0	78.0	1.929
1.4708	28.0	77.0	1.980
1.0715	20.0	72.0	1.855
1.2030	23.0	73.0	1.964
1.3077	25.0	77.0	1.882
1.3826	26.0	78.0	1.870
1.4885	28.0	80.0	1.849
1.0984	21.0	74.0	1.861
1.1845	22.0	73.0	1.876
1.3216	24.0	73.0	1.900
1.3820	26.0	76.5	1.904
1.4773	28.0	80.0	1.881

Av. result: 1.904 mg. K/1.375 Gm.
Range: 2.012 − 1.831 = 0.181 or 9.5%.
Standard deviation: 0.046 or 46/1904 × 100 = 2.4%

DISCUSSION

This procedure has been used in this laboratory over a period of about three years and more than a thousand determinations have been made with it on mixtures representing variants of that described above and containing in some instances, ingredients foreign to the formula given, e. g., multivitamin-antibiotic formulations. The simplicity and precision of the procedure allow its practical application to low cost control of menadione (vitamin K) formulations.

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A Study of the Inhibitory Concentrations of Glycerin-Sorbitol and Propylene Glycol-Sorbitol Combinations on the Growth of Microorganisms*

By MARTIN BARR and LINWOOD F. TICE

Glycerin and sorbitol were found to supplement each other in their inhibition of both molds and bacteria. It is believed that this inhibitory action is purely one of osmotic effect. Propylene glycol appears to have a specific inhibitory effect against bacteria and most molds; the addition of 10% w/v propylene glycol was found to inhibit the growth of these organisms. Thirty per cent w/v propylene glycol is required to inhibit *Monilia albicans.* Studies have indicated that the inhibition produced by the addition of propylene glycol to sorbitol solutions is not purely the result of osmotic effect, as is the case with glycerin, but probably one related to its chemical structure. It may, therefore, be concluded that propylene glycol has a significant and useful preservative activity in many preparations including those made with sorbitol.

SORBITOL is a hexahydric alcohol having the empirical formula $C_6H_{14}O_6$ and a molecular weight of 182.2. It has found increasing use in product formulation as a humectant, solvent, bodying agent, lubricant, softener, plasticizer, and blending agent. Most sugars and polyols commonly employed in product formulation including glycerin, sucrose, invert sugar, and sorbitol support the growth of microorganisms. It, therefore, becomes necessary to include a preservative in preparations containing these substances in order to prevent spoilage. This paper reports the results of a study to determine those ratios and concentrations of glycerin and sorbitol and propylene glycol and sorbitol that are inhibitory to the growth of various microorganisms. A subsequent paper reports the effectiveness of some commonly used preservatives in preventing growth in dilute sorbitol solutions where the osmotic pressure is itself not sufficiently great to restrain growth.

EXPERIMENTAL

Solutions of sorbitol[1] in glycerin-water[2] and sorbitol in propylene glycol-water[3] were prepared containing glycerin or propylene glycol in a concentration of 0 to 70% w/v using gradations of 10%. All solutions were so adjusted with 0.1 *N* NaOH that the final pH after sterilization (121.3° for 20 minutes) was 7.4. An inoculum of 0.1 ml. of each test organism in beef heart infusion was introduced into 10 ml. of each of the sterile solutions contained in test tubes. All inoculations were made in quadruplicate. Two tubes were then placed in the incubator (37°) and the other two allowed to stand at room temperature (25° ± 2°). The tubes were observed at frequent intervals for a period of thirty days for evidence of growth. The test organisms employed in this study were: *Pseudomonas aeruginosa,*[4] *Staphylococcus aureus,*[5] *Bacillus subtilis,*[6] *Aspergillus niger,*[7] *Penicillium notatum,*[8] and *Monilia albicans.*[9]

RESULTS AND DISCUSSIONS

The inhibitory concentrations of the glycerin-sorbitol and propylene glycol-sorbitol combinations are listed in Tables I and II.

The results in Table I indicate that molds are more difficult to restrain in combinations of sorbitol and glycerin than are bacteria. The addition of 20–30% w/v glycerin to 10% sorbitol solution was required to raise the osmotic pressure sufficiently to inhibit the growth of all bacteria studied. Molds were not inhibited until 50% w/v glycerin was present in the solution containing 10% sorbitol. As the concentration of sorbitol in the solution increases, the strength of glycerin required for the combination to restrain growth is lowered, as might be expected. It is apparent that this inhibitory action is purely one of osmotic effect.

The results in Table II indicate also that molds are more difficult to restrain with sorbitol and propylene glycol combinations than are bacteria. *Monilia albicans* was found to be the most difficult mold to inhibit. As little as 10% w/v propylene glycol added to sorbitol solutions of low concentration resulted in the inhibition of bacteria and most molds. Thirty per cent w/v propylene glycol was required

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[1] Sorbitol, used in the form of Sorbo,® a 70% w/w sorbitol solution, Atlas Powder Company.
[2] Glycerin, U. S. P. XIV, Colgate-Palmolive-Peet Company.
[3] Propylene glycol, Carbide and Carbon Chemicals Company.
[4] *Pseudomonas aeruginosa*, isolated from a patient at the University of Pennsylvania Hospital.
[5] *Staphylococcus aureus*, FDA 209, A.T.C.C. No. 6538.
[6] *Bacillus subtilis*, obtained as an isolated contaminant from air in the laboratory of the Department of Bacteriology, Philadelphia College of Pharmacy and Science.
[7,8] *Aspergillus niger* and *Penicillium notatum*, isolated from contaminated products in the laboratory of the Department of Pharmacy, Philadelphia College of Pharmacy and Science.
[9] *Monilia albicans*, isolated from a patient at Children's Hospital, Philadelphia.

TABLE I.—THE INHIBITORY CONCENTRATIONS OF GLYCERIN-SORBITOL SOLUTIONS[a]

% w/v Sorbitol in Solution	% w/v Glycerin in Solution —Organisms—					
	S. aureus	*B. subtilis*	*P. aeruginosa*	*Aspergillus niger*	*Penicillium notatum*	*M. albicans*
0	40	40	30	50	50	50
10	20	30	20	50	50	50
20	20	20	10	40	40	50
30	10	10	0	40	40	30
40	10	0	0	40	30	30
50	0	0	0	20	20	30
60	0	0	0	20	0	30
70	0	0	0	20	0	20

[a] The following example is given to clarify the data presented in Table I. From this table, line 2, it will be seen that a solution containing 10 per cent sorbitol and 20 per cent glycerin restrains the growth of *S. aureus* while 10 per cent sorbitol and 50 per cent glycerin is required for *Aspergillus niger*.

TABLE II.—THE INHIBITORY CONCENTRATIONS OF PROPYLENE GLYCOL-SORBITOL SOLUTIONS[a]

% w/v Sorbitol in Solution	% w/v Propylene Glycol in Solution —Organisms—					
	S. aureus	*B. subtilis*	*P. aeruginosa*	*Aspergillus niger*	*Penicillium notatum*	*M. albicans*
0	10	10	10	10	10	30
10	10	10	10	10	10	30
20	10	10	10	10	10	30
30	10	10	0	10	10	30
40	10	10	0	10	10	30
50	0	0	0	10	10	30
60	0	0	0	10	0	30
70	0	0	0	10	0	30

[a] The following example is given to clarify the data presented in Table II. From this table, line 2, it will be seen that a solution containing 10 per cent sorbitol and 10 per cent propylene glycol restrains all test organisms except *M. albicans* in which case 30 per cent propylene glycol and 10 per cent sorbitol are required.

to inhibit *Monilia albicans* in such solutions. These results indicate that the inhibition produced by the addition of propylene glycol to sorbitol solutions is not purely the result of its osmotic effect but probably one related to the free methyl group in its chemical structure. It may, therefore, be concluded that propylene glycol has a significant and useful preservative activity in solutions of sorbitol.

The fact that propylene glycol has preservative properties when added to other polyol solutions is of great importance since it may be both advantageous and necessary to combine such polyols. Propylene glycol may also be used to advantage for its solvent properties since it will dissolve many substances even better than does glycerin. If only propylene glycol is used in formulations, one encounters objections based on its taste. A combination of sorbitol and propylene glycol may well solve both difficulties, each polyol complementing the other.

SUMMARY

1. The required concentrations of glycerin and sorbitol in combination and those of propylene glycol and sorbitol to restrain microorganisms was studied.

2. Molds were found to be more difficult to inhibit than bacteria in aqueous sorbitol-glycerin and sorbitol-propylene glycol solutions.

3. A combination of 20–30% w/v glycerin and 10% sorbitol was necesaary to inhibit the bacteria studied, while a combination of 50% w/v glycerin and 10% sorbitol was necessary to inhibit the molds. As the concentration of sorbitol was increased, the concentration of glycerin required was diminished. The inhibitory action of these combined polyols is apparently purely one of osmotic effect.

4. As little as 10% w/v propylene glycol added to sorbitol solutions of low concentration resulted in the inhibition of bacteria and most molds, but a concentration of 30% w/v propylene glycol was required to inhibit *Monilia albicans* in such solutions. The preservative activity of propylene glycol is probably one related to its chemical structure.

5. Propylene glycol has a significant and useful preservative activity in sorbitol solutions.

A Study of the Inhibitory Concentrations of Various Sugars and Polyols on the Growth of Microorganisms*

By MARTIN BARR and LINWOOD F. TICE

A comparison of the inhibitory concentrations of various sugars and polyols on the growth of various organisms has been made. Glycerin was found to have a lower inhibitory concentration than levulose and dextrose. Sorbitol and invert sugar inhibit the growth of organisms in a concentration lower than that required with sucrose. The inhibitory effect of each substance is believed to be largely one of osmotic pressure.

GLYCERIN, levulose, dextrose, sorbitol, sucrose, and invert sugar are widely used by the pharmaceutical, cosmetic, and candy industries. Each of these sugars or polyols is a good nutrient for microorganisms and the spoilage of preparations containing them has long been recognized as a troublesome problem. Because of this, it was deemed advisable to determine the concentrations of these substances, alone and in combination, which are inhibitory for some of the microorganisms commonly recognized as being responsible for this spoilage. This paper deals with the results of such a study.

EXPERIMENTAL

The following sugars and polyols were included in this study: glycerin,[1] levulose,[2] dextrose,[3] sorbitol,[4] sucrose,[5] invert sugar,[6] Nulomoline®,[7] a mixture of 90 parts of sucrose and 7 parts of sorbitol, a mixture of 80 parts of sucrose and 14 parts of sorbitol, a mixture of 90 parts of sucrose and 7 parts of Nulomoline, and a mixture of 80 parts of sucrose and 14 parts of Nulomoline.

Solutions of the substances under investigation were studied for their inhibitory activity against organisms in gradations of 10% at first and, then, at 5%. All solutions were prepared on a % w/w basis and were adjusted to a pH of 5.6 using 0.1 *N* NaOH or 0.1 *N* HCl. This hydrogen-ion concentration was decided upon as it was found to be the average for the aqueous polyol and sugar solutions. Ten milliliters of each solution was transferred into test tubes and the solutions were sterilized in the autoclave at 121.3° for 20 minutes. The sterile solutions were then inoculated with a 0.1 ml. beef heart infusion suspension of the five individual organisms found in other work to be among the major sources of trouble in the development of growth on sugar and polyol solutions. The organisms were *Staphylococcus aureus*,[8] *Pseudomonas aeruginosa*,[9] *Penicillium notatum*,[10] *Aspergillus niger*,[11] and *Monilia albicans*.[12] All inoculations were made in quadruplicate and each series of tests repeated three times. The inoculated tubes were then placed in an incubator (37°) and were observed at frequent intervals for a period of thirty days for evidence of growth.

RESULTS AND DISCUSSIONS

The observed inhibitory concentrations of the sugar and polyol solutions and some of their combinations are listed in Table I. Results indicate that molds are more difficult to inhibit than bacteria and that the inhibitory concentrations of the substances studied are related to their molecular weights. The inhibitory effect is probably largely—if not solely—one of osmotic pressure. Those substances with a low molecular weight have a greater effect than substances with a higher molecular weight. Thus, glycerin (mol. wt. 92.09) has a lower inhibitory concentration than levulose (mol. wt. 180.16) and dextrose (mol. wt. 180.16). Sorbitol (mol. wt. 182.17) and invert sugar (mol. wt. 360.32 ÷ 2) inhibit the growth of organisms in a concentration lower than that required of sucrose (mol. wt. 342.30).

The results obtained with Nulomoline and invert sugar are very interesting. Both of these substances have similar inhibitory concentrations, as might be expected since Nulomoline is an invert sugar preparation. However, the inhibitory concentration of these invert sugar solutions is less than that required with sucrose. This is not surprising, although the opposite is generally considered to be true by many workers. Although the molecular weight of invert sugar is greater than that of sucrose (360.32 to 342.30), it must be remembered that invert sugar is a mixture of two individual sugars, levulose and dextrose, each having a molecular weight of 180.16. Therefore, there are a greater number of moles in a given invert sugar solution than in a sucrose solution of the same w/w strength. The osmotic effect of invert sugar solutions is,

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[1] Glycerin, U. S. P. XIV, Colgate-Palmolive-Peet Company, containing 98.74% w/w $C_3H_8O_3$.
[2] Levulose, C. P., Pfanstiehl Laboratories.
[3] Dextrose, Anhydrous, Merck Analytical Reagent.
[4] Sorbitol, Crystalline D-Sorbitol, Atlas Powder Company, containing a minimum polyhydric alcohol content of 99%.
[5] Sucrose, U. S. P., Sunny Cane Extra Fine Granulated, McCahan Sugar Refinery, Philadelphia, Pennsylvania.
[6] Invert sugar, prepared by mixing together equal parts of levulose and anhydrous dextrose.
[7] Nulomoline, a brand of invert sugar, American Molasses Company, Brooklyn, New York.
[8] *Staphylococcus aureus*, F.D.A. 209, A.T.C.C. No. 6538.
[9] *Pseudomonas aeruginosa*, isolated from a patient at the University of Pennsylvania Hospital.
[10,11] *Penicillium notatum* and *Aspergillus niger*, isolated from contaminated products in the laboratories of the Philadelphia College of Pharmacy and Science.
[12] *Monilia albicans*, isolated from a patient at Children's Hospital, Philadelphia.

TABLE I.—INHIBITORY CONCENTRATIONS (% w/w) OF SUGARS, POLYOLS, AND SOME OF THEIR COMBINATIONS

Substance	Mol. Wt.	Test Organism				
		S. aureus	*Pseudomonas aeruginosa*	*Penicillium notatum*	*Aspergillus niger*	*Monilia albicans*
Glycerin	92.09	30	25	30	40	35
Levulose	180.16	30	30	50	[a]	50
Dextrose	180.16	30	30	50	[a]	50
Sorbitol	182.17	45	40	55	60	60
Sucrose	342.30	55	50	65	65	65
Invert Sugar	360.32[b]	45	40	55	60	60
Nulomoline	360.32	45	50	55	60	60
Sucrose, 90 parts		51.03	46.40	60.31	60.31	60.31
Sorbitol, 7 parts		3.97	3.60	4.69	4.69	4.69
Total	330.74	55	50	65	65	65
Sucrose, 80 parts		51.03	46.40	55.32	55.32	55.32
Sorbitol, 14 parts		3.97	3.60	9.68	9.68	9.68
Total	318.40	55	50	65	65	65
Sucrose, 90 Parts		46.40	46.40	55.67	60.31	55.67
Nulomoline, 7 Parts		3.60	3.60	4.33	4.69	4.33
Total	330.74	50	50	60	65	60
Sucrose, 80 Parts		38.30	31.21	46.81	55.32	51.07
Nulomoline, 14 Parts		6.70	8.79	8.19	9.68	8.93
Total	318.40	45	40	55	65	60

[a] Solutions of a concentration of 55% w/w crystallize out upon standing; solutions of a concentration of 50% w/w do not inhibit the growth of *Aspergillus niger*.
[b] Although the molecular weight of invert sugar is listed as 360.32, a mole of this substance consists of one mole each of dextrose and levulose, each having a molecular weight of 180.16.

therefore, greater than that of sucrose and, thus, since osmotic activity is related to mole concentration, invert sugar solutions may be expected to restrain organisms at a lower concentration than is required with sucrose.

Studies on the sucrose-sorbitol solutions indicate that such solutions are similar in inhibitory effect to those of sucrose alone. It must be remembered that the highest proportion of sorbitol in these solutions was only 14 parts of sorbitol to 80 parts of sucrose. The osmotic pressure of such solutions, therefore, is only slightly above that of sucrose alone. This increase in osmotic pressure is not sufficient to alter the inhibitory concentration of the sorbitol-sucrose solutions from those of sucrose alone. If the proportion of sorbitol were increased further, it would be expected that the inhibitory concentration required would be lower.

Solutions containing the sucrose-Nulomoline combination were found to have a slightly lower inhibitory concentration in some cases than either those with sucrose alone or those with sucrose and sorbitol. Insofar as the Nulomoline effect is concerned, its presence would be expected to reduce the overall concentration required by reason of the increased osmotic effect. The reason for the slight superiority of Nulomoline over sorbitol is not understood since, gram for gram, these two materials have about the same osmotic effect. In this connection, it should be remembered that the gradations in concentration in these experiments did not go below 5% and it is quite possible that these two materials are actually much closer in their inhibitory effects than the tabulated results would seem to indicate.

Observations on the sucrose solutions were also of interest. It is shown in Table I that sucrose in a concentration of 65% w/w inhibits the growth of molds. However, upon standing for a long period of time (more than 30 days), mold growth was observed in sucrose solutions of this strength. In effect, it is believed impossible to inhibit organisms—especially molds—in sucrose solutions that are unpreserved for a long period of time. This is in accordance with observations on Simple Syrup, U. S. P. XV, which is a 64.7% w/w solution of sucrose in purified water. Syrups prepared in proper strength are stable for a period of time. However, upon standing, they tend to become slightly brown in color (inversion) and show mold growth. In reality, it is almost impossible to prepare Syrup, U. S. P. XV, which will remain free of mold growth for a long period of time unless aseptic technique is employed.

The following is believed to be the explanation of this behavior. A saturated sucrose solution will not permit mold growth, not solely because of osmotic pressure but because the organisms present cannot utilize sucrose without first hydrolyzing it to monosaccharide sugar. This, they cannot do in a saturated solution since the available water is so firmly bound to the sucrose molecules. Gradually, however, some inversion takes place and monosaccharide sugar is formed. This the organisms can utilize directly without hydrolysis and they proceed to multiply in spite of the fact that some slight increase in osmotic pressure has taken place by the inversion of the sucrose. The validity of this hypothesis is now being tested experimentally.

SUMMARY

1. A study of the inhibitory concentrations of various sugars and polyols in aqueous solution using five organisms, commonly recognized as being responsible for spoilage in products containing these substances, has been carried out.

2. The inhibitory concentrations of the sugars and polyols were found to be related to their molecular weights and the inhibitory effect is believed largely one of osmotic effect.

3. Glycerin was found to have a lower inhibitory concentration than levulose and dextrose; sorbitol and invert sugar were found to have a lower inhibitory concentration than sucrose.

4. A hypothesis is presented which is believed to explain the lower inhibitory concentration required with invert sugar as compared with sucrose and the growth of molds in concentrated sucrose solutions upon standing for a long period of time.

The Preservation of Aqueous Sorbitol Solutions*

By MARTIN BARR and LINWOOD F. TICE

A study of the effect of pH on the inhibitory concentrations of sorbitol and a study of the effectiveness of various preservatives, suitable for internal use, in preventing the growth of organisms in sorbitol solutions have been carried out. From a practical viewpoint, although pH does have some influence on the inhibitory effect of solutions of sorbitol, it alone cannot insure against the growth of certain organisms. Fifteen preservatives and preservative combinations were studied for their effectiveness in sorbitol solutions. It was found that, of these, only a combination of methylparaben (0.12% w/v) and propylparaben (0.02% w/v), and sorbic acid (0.15% w/v) were generally effective in preserving aqueous sorbitol solutions. In the case of sorbic acid, an occasional failure to protect the solution was observed.

SORBITOL is a hexahydric alcohol having the formula $C_6H_{14}O_6$. It is prepared by the catalytic hydrogenation of dextrose. Sorbitol (D-sorbitol) is a white, odorless, crystalline powder having a faint, sweet, cooling, and pleasant taste. It has found increased use in various products because of its many desirable properties, among which are its humectant, solubilizing, blending, and bodying actions. It is stable in the dry state or in sterile aqueous solution. It is not attacked by dilute acids or alkalies, is not subject to hydrolysis, and does not darken.

Similar to other polyols and sugars used in product formulation, sorbitol supports the growth of organisms. High concentrations of such substances are necessary to inhibit growth, especially that of molds (1). Since the inhibiting concentration required functions largely by virtue of its osmotic activity, it is usually much too high to be suitable in most pharmaceutical products, regardless of the sugar or polyol employed. It is necessary, therefore, to add a preservative to preparations containing these substances in order to prevent spoilage. In this connection, the advantage of using propylene glycol in combination with sorbitol or other polyols in order to prevent the growth of organisms has been suggested by the authors (2).

The experimental work reported in this paper deals with a study of the effect of pH on the inhibitory concentrations of sorbitol and a study of the effectiveness of various preservatives, suitable for internal use, in preventing the growth of various organisms in sorbitol solutions.

EXPERIMENTAL

Effect of pH on the Inhibitory Concentrations of Sorbitol.—Solutions of sorbitol[1] in water were prepared in concentrations ranging from 10 to 70% w/v, using gradations of 10%. Solutions of similar concentrations were prepared at a pH ranging from 2 to 8, in gradations of 1 pH unit. McIlvane's buffer (3) was used to obtain the various hydrogen-ion concentrations. An inoculum of 0.1 ml. was introduced into 10 ml. of each of the sterile sorbitol solutions previously autoclaved at 121.3° for 20 minutes. The inoculum consisted of a 24-hour suspension of the test organism in beef heart infusion. The organisms which were included in this study were:

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[1] Crystalline D-sorbitol, Atlas Powder Company, Wilmington, Del.

Staphylococcus aureus,[2] *Bacillus subtilis*,[3] *Pseudomonas aeruginosa*,[4] *Monilia albicans*,[5] *Aspergillus niger*,[6] and *Penicillium notatum*.[7] All inoculations were made in quadruplicate. Two tubes were placed in the incubator (37°) and the other two, allowed to stand at room temperature (25° ± 2°). The tubes were observed at frequent intervals for a period of thirty days for evidence of growth. The results of this study are expressed in Table I.

TABLE I.—EFFECT OF pH ON THE INHIBITORY CONCENTRATIONS OF SORBITOL

Organisms	% Sorbitol w/v Inhibiting Growth pH of Media 2	3	4	5	6	7	8
Staphylococcus aureus	[a]	—	50	50	50	50	50
Pseudomonas aeruginosa	—	—	40	40	40	40	40
Bacillus subtilis	—	—	50	50	50	50	50
Monilia albicans	70	70	70	70	70	70	70
Aspergillus niger	—	70	70	70	70	70	70
Penicillium notatum	—	—	60	60	60	60	60

[a] —No evidence of growth.

cetylpyridinium chloride,[8] benzethonium chloride,[9] cetyltrimethylammonium bromide,[10] benzalkonium chloride,[11] DHA-S,[12] and sorbic acid.[13] The effectiveness of these substances in preventing the growth of organisms in 20% w/v sorbitol solutions was determined. The organisms employed were the same as those used in the study of the effect of pH on the inhibitory concentrations of sorbitol. It was observed that the concentration of sorbitol in solution did not materially influence the overall effectiveness of a preservative, i. e., if a given substance prevented growth in a 20% w/v sorbitol solution, it also prevented growth in a sorbitol solution of any strength. The 20% w/v strength was chosen because sorbitol in this concentration is not inhibitory to the growth of organisms and the results using it would be indicative of what might be expected in any solution containing sorbitol.

An inoculum of 0.1 ml. was introduced into 10 ml. of the sterile[14] solutions previously autoclaved at 121.3° for 20 minutes. The inoculum consisted of a 24-hour suspension of the individual organisms in thioglycollate medium. All inoculations were made in quadruplicate. Two tubes were placed in the incubator (37°) and the other two, allowed to stand at room temperature (25° ± 2°). The tubes were observed at frequent intervals for a period of thirty days for evidence of growth. The results are reported in Table II.

TABLE II.—EFFECTIVENESS OF PRESERVATIVES IN AQUEOUS SORBITOL SOLUTIONS

Preservative	Concentration % w/v or v/v	Organisms: *S. aureus*	*B. subtilis*	*Ps. aeruginosa*	*M. albicans*	*Asp. niger*	*Pen. notatum*
Sodium benzoate	0.10	—[b]	—	+[a]	+	+	+
Sodium benzoate	0.50	—	—	+	+	+	+
Benzoic acid	0.20	—	—	+	+	—	—
Benzoic acid	0.40	—	—	+	+	—	—
Methylparaben	0.18	—	—	—	±[c]	—	—
Propylparaben	0.05	—	—	+	—	—	—
Butylparaben	0.05	—	+	—	±	—	—
Ethylparaben	0.10	—	—	—	±	—	—
Methylparaben Propylparaben	0.12 0.02	—	—	—	—	—	—
Cetylpyridinium chloride	0.10	+	+	+	+	+	+
Benzethonium chloride	0.10	—	+	+	+	+	+
CTAB	0.10	+	+	+	+	+	+
Benzalkonium chloride	0.10	—	+	+	+	+	+
DHA-S	0.40	—	—	—	—	+	+
Saligenin	0.10	—	—	—	+	+	+
Alcohol	10.00	—	—	—	+	—	—
Alcohol	20.00	—	—	—	+	—	—
Sorbic acid	0.15	—	—	±	±	±	±
Control	—	+	+	+	+	+	+

[a] + Growth observed within 30 days.
[b] — No growth observed within 30 days.
[c] ± Very slight growth observed.

Effectiveness of Preservatives in Sorbitol Solution.—Various concentrations of the following substances were employed in this study: sodium benzoate, benzoic acid, methylparaben, ethylparaben, butylparaben, a combination of methylparaben and propylparaben, saligenin, alcohol,

[2] *Staphylococcus aureus*, FDA 209, A.T.C.C. No. 6538.
[3] *Bacillus subtilis*, isolated as an air contaminant from the Bacteriology Laboratory, Philadelphia College of Pharmacy and Science.
[4] *Pseudomonas aeruginosa*, isolated from a patient at the University of Pennsylvania Hospital.
[5] *Monilia albicans*, isolated from a patient at Children's Hospital, Philadelphia.
[6,7] *Aspergillus niger* and *Penicillium notatum*, isolated from contaminated products in the laboratories of the Philadelphia College of Pharmacy and Science.

[8] Ceepryn® chloride, Wm. S. Merrell Company, Cincinnatti, Ohio.
[9] Phemerol® chloride, Parke, Davis & Company, Detroit, Mich.
[10] CTAB, Fine Organics, Inc., New York, N. Y.
[11] Zephiran® Chloride, Winthrop Laboratories, New York, N. Y.
[12] DHA-S, dehydroacetic acid, sodium salt, Dow Chemical Company, Midland, Mich.
[13] Sorbic acid, 2, 4-hexadienoic acid, Carbide and Carbon Chemicals Company, New York, N. Y.
[14] Solutions containing alcohol were prepared aseptically.

RESULTS AND DISCUSSION

It may be observed from the results in Table I that the bacteria employed did not grow in sorbitol solutions having a pH below 4. Although there was evidence of growth throughout the remainder of the pH range tested, maximum development was noted at pH 5–7. Growth was less at pH 4–5 and only slight at pH 8.

Monilia albicans grew throughout the entire pH range: maximum growth being observed at pH 4–7; lesser growth, at pH 2–3; and very slight growth, at pH 8.

Aspergillus niger and *Penicillium notatum* did not grow at pH 2; the former did grow at pH 3, the latter did not. Maximum growth of these molds was at pH 5–7, slight growth being observed at pH 8.

Of particular interest is the fact that the molds grew in very acid solutions and were not inhibited by rather high concentrations of sorbitol. Therefore, it may be concluded that, although pH does have some influence on the inhibiting effect of solutions of sorbitol, it alone cannot insure against the growth of organisms. It must be recognized that the buffer salts which were used to obtain the various pH values may in themselves inhibit or support the growth of organisms and that results listed in Table I may be somewhat different if substances other than citric acid and disodium phosphate (McIlvane's buffer salts) are used for buffering purposes.

It should be mentioned that experiments have been carried out on the effect of pH on the growth of the organisms studied in beef heart infusion and that results similar to those obtained with sorbitol solutions were observed.

Examination of Table II reveals that sodium benzoate, benzoic acid, propylparaben, methylparaben, ethylparaben, butylparaben, cetylpyridinium chloride, benzethonium chloride, cetyltrimethylammonium bromide, benzalkonium chloride, DHA-S, alcohol, and sorbic acid were not capable of inhibiting the growth of all the test organisms.

A combination of 0.12% w/v methylparaben and 0.02% w/v propylparaben was found to be effective in inhibiting the growth of all the organisms tested. It is generally reported that the parabens are more effective in inhibiting the growth of yeast and mold rather than bacteria. Nevertheless, in this study, methylparaben and propylparaben in combination inhibited the bacteria as well as the molds.

Sorbic acid has been reported to be a good fungistatic agent for foods (4, 5) and pharmaceuticals (6). In our studies, sorbic acid did not completely protect all the sorbitol solutions against growth. There were a few instances where very slight growth occurred following inoculations with *Pseudomonas aeruginosa* and the molds. In general, sorbic acid usually was effective in preserving the sorbitol solutions; some few failures were, however, observed.

It is of interest to note that 10% v/v alcohol inhibited the growth of all organisms with the exception of *Monilia albicans*. Increasing the concentration of the alcohol to 20% v/v failed to inhibit this organism.

SUMMARY

1. A study of the effect of pH on the inhibitory concentrations of sorbitol and of the effectiveness of various preservatives in preventing organism growth in sorbitol solutions has been carried out.

2. Bacteria did not grow in sorbitol solutions having a pH below 4; *Monilia albicans* grew throughout the entire pH range of 2–8; *Aspergillus niger* did not grow below a pH of 3; and *Penicillium notatum* was inhibited in solutions below a pH of 4.

3. From a practical preservation viewpoint, although pH does have some influence on the inhibitory effect of solutions of sorbitol, it alone cannot insure against organism growth.

4. Of the various preservatives studied, a combination of 0.12% w/v methylparaben and 0.02% w/v propylparaben was the most effective in preventing organism growth in sorbitol solutions.

5. Sorbic acid, 0.15%, usually was effective in preserving sorbitol solutions although some occasional failures were noted.

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The Preparation of Azopyrimidines and Their Metallized Derivatives*

By WILLIAM O. FOYE† and J. GEORGE JEFFREY‡

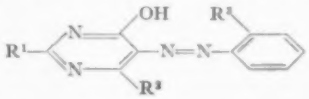
A number of *o*-hydroxyazopyrimidines has been prepared for anticancer testing. These compounds were obtained by direct coupling into the pyrimidine ring, coupling of a diazotized aminopyrimidine into a phenol ring, or coupling of a diazotized aminophenol with acetoacetic ester followed by ring closure with urea or thiourea. Both spectral and chemical evidence suggest that these compounds exist more as quinone hydrazones than as azo compounds. The *o*-hydroxyazopyrimidines chelated heavy metal ions but did not show appreciable activity against Sarcoma 180.

RECENT ATTEMPTS to find possible antagonists to the formation of nucleic acids in the animal have led to the synthesis of numerous pyrimidine derivatives. Anticancer tests on these compounds have revealed growth-retarding effects on tumors from a number of them, notably 6-mercaptopurine and 8-azaguanine (1). 2-Thiouracil has also shown ability to decrease incidence of liver cancer, while uracil itself overcomes this inhibition (2). Since a number of azo compounds have shown both cancer-producing and cancer-inhibiting activities (3), a series of azo derivatives of some pyrimidines, mainly uracil and substituted uracils, has been synthesized and tested for cancer chemotherapeutic action. In addition, these azo compounds were prepared having hydroxy groups *ortho* to the azo linkage, thereby making them theoretically capable of chelating heavy metals. This should provide an opportunity for testing the effect of a metal-chelating agent on cancer growth; it has been suggested by Boyland and Watson (4), for instance, that the carcinogenic effects of various *o*-aminophenols may be due to metal chelation *in vivo*.

DISCUSSION

Methods of Synthesis.—The preparation of the azopyrimidines was first attempted by the direct coupling of a diazonium salt with uracil or its relatives. The compound 5-phenylazouracil had been prepared previously by Bogert and Davidson (5) from isodialuric acid and phenylhydrazine, but no melting point was listed; direct coupling of benzenediazonium chloride with uracil gave a product of the same color as that reported which also showed no melting below 300°. A correct analysis for carbon-hydrogen was not obtained, however. Coupling of uracil with diazotized *o*-aminophenol also gave a product which could not be purified and which appeared to be light-sensitive. Polonovski and Pesson (6) have shown that benzenediazonium chloride will couple in the 5-position with pyrimidines having one or two hydroxy groups, but they listed no physical constants for 5-phenylazouracil.

Preparation of this type of compound was successfully carried out by a ring closure. Diazotized *o*-aminophenol was coupled with acetoacetic ester, and the product was condensed with urea to form the pyrimidine ring. This procedure was based on a method used by Andersag and Westphal (7). The azopyrimidine thus formed, 5-*o*-hydroxyphenylazo-6-methyluracil (structure I*a*), was obtained in a yield of 44%. Similarly, using thiourea instead of urea, the corresponding sulfur compound (I*b*) was obtained. Purification of these compounds was achieved by continuous extraction with Skellysolve® B for periods of thirty-six hours or more.



Structure I.—Phenylazopyrimidines: (*a*), R^1=OH, R^2=CH_3, R^3=OH; (*b*), R^1=SH, R^2=CH_3, R^3=OH; (*c*), R^1 and R^2=OH, R^3=H; (*d*), R^1, R^2 and R^3=OH.

A third procedure employed for preparing azopyrimidines involved the diazotization of 5-aminouracil or 5-amino-6-methyluracil followed by coupling with β-naphthol. The naphthylazopyrimidines (structure II) thus formed could be purified by continuous extraction with water for extended periods. The 1-uracilyl-5′-azo-2-naphthol so obtained decomposed with effervescence at 297–298°, an elevation of about 10° over the decomposition point reported by Bogert and Davidson (5) for this product.

An impression concerning the instability of these azopyrimidines may be gained from a review of the purification measures which were attempted and led to decomposed products. With compound II*b*, for example, which melted at 273–275° after continuous extraction with water, recrystallization from absolute ethanol gave a product which decomposed with effervescence at 239°; recrystallization from water

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† Present address: Massachusetts College of Pharmacy, Boston, to which any inquiries should be sent.

‡ On leave of absence from the College of Pharmacy, University of Saskatchewan, Saskatoon, 1953–1954.

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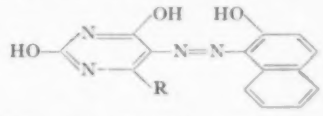
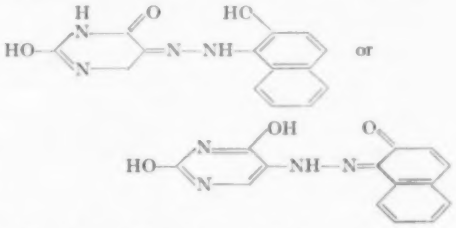
resulted in hydrolysis to β-naphthol; and repeated solution in 10% sodium hydroxide solution followed by precipitation with acetic acid also resulted in formation of β-naphthol. The facility with which these compounds break down suggested an analogy with the highly-reactive anticancer agents of the triethylenemelamine type. Diazotized 5-aminouracil was also coupled with uracil itself to give azouracil-5,5′, which should be even less stable than the phenyl or naphthyl azo compounds. Bogert and Davidson (5) reported a preparation of this compound, but were unable to obtain a correct analysis for it. We obtained an approximate carbon-hydrogen analysis for this compound which should suffice, in view of the known difficulty of purification of azo compounds in general and of the instability of these in particular.



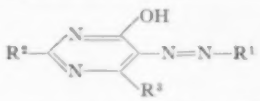
Structure II.—Naphthylazopyrimidines: (*a*), R = H; (*b*), R = CH_3.

Although direct coupling of a diazonium salt into the uracil nucleus did not give pure azo compounds, coupling into barbituric acid [postulated to be 2,6-diketo-4-hydroxypyrimidine by Arndt (8)] gave azo compounds that showed correct analyses. 5-Phenylazobarbituric acid (I*c*), which was prepared, has been shown to be identical in properties and melting point with alloxan phenylhydrazone by Kuhling (9); hydrolysis, a tendency not generally shown by the azo linkage. A comparison of the absorption spectra of the azopyrimidines with those of the phenylazonaphthols lends further support to this possibility. As shown in Table II, the phenylazopyrimidines have absorption maxima in agreement with that of alloxan phenylhydrazone, while 1-uracilyl-5′-azo-2-naphthol has a maximum agreeing closely with that of 1-phenylazo-2-naphthol, previously shown by Kuhn and Bär (10) to exist mainly as the quinone hydrazone. In contrast, 4-phenylazo-1-naphthol was shown (10) to exist as a mixture of azo and hydrazone structures in neutral media. A consistent bathochromic shift in maxima may also be noted with the addition of *o*-hydroxy groups in each case, a structural factor which could possibly favor the formation of the quinone hydrazone structure. Structure III illustrates the possibilities for a hydrazone structure that exists with 1-uracilyl-5′-azo-2-naphthol.



Structure III.—Quinone hydrazone forms of 1-uracilyl-5′-azo-2-naphthol.

TABLE I.—AZOPYRIMIDINES



R^1	R^2	R^3	M. p., °C.[a]	Yield, %[b]	Extn. solvent	Formula	Analyses, %— Calcd.	Found[c]
o-Hydroxyphenyl	OH	OH	269–273 (*d*)	55	Skelly B	$C_{10}H_8O_4N_4$	C: 48.38	48.17
							H: 3.25	3.38
2-Hydroxy-1-naphthyl	OH	CH_3	273–275 (*d*)	35	Water	$C_{15}H_{12}O_3N_4$	C: 60.80	59.15
							H: 4.08	4.70
o-Hydroxyphenyl	SH	CH_3	153–157 (*d*)	49	Skelly B	$C_{11}H_{10}O_2N_4S$	C: 50.37	50.18
							H: 3.84	3.80
o-Hydroxyphenyl	OH	CH_3	194 (*d*)	44	Skelly B	$C_{11}H_{10}O_3N_4$	C: 53.66	54.67
							H: 4.09	4.43
5-Uracilyl	OH	H	280 (*d*)	27	Skelly B	$C_8H_6O_4N_6$	C: 38.40	37.04
							H: 2.42	2.81

[a] The melting points are uncorrected; (*d*) = decomposition, generally accompanied by effervescence.
[b] The yield is calculated for either the final coupling or ring-closure step.
[c] The analyses were done by the Clark Microanalytical Laboratory, Urbana, Ill. In some instances a special catalyst was required to insure complete combustion.

this was further supported by our finding of identical absorption spectra for both compounds. The products obtained from this coupling procedure are shown in Structures I*c* and I*d*. The physical constants of the new azopyrimidines prepared are listed in Table I.

Structural Considerations.—The possibility that the azopyrimidines synthesized may exist as quinone hydrazones rather than as azo compounds seemed likely, particularly in view of their ease of

Chelation with Metals.—Metallization reactions were carried out on several of the *o*-hydroxyazopyrimidines, and evidence for chelation was noted in each case. The reactions were accomplished in either aqueous alkali, alkaline ethylene glycol, or triethanolamine with iron, nickel, and cobalt ions, and were continued until only one band of a different color than the starting material was present on a paper chromatogram. The evidence for chelation consisted of a marked color change, a drop in pH dur-

TABLE II.—ABSORPTION SPECTRA OF AZOPYRIMIDINES AND AZONAPHTHOLS

Compound	λ max (mμ)[a]
5-Phenylazouracil	345
Alloxan phenylhydrazone	380
5-Phenylazobarbituric acid	380
5-*o*-Hydroxyphenylazobarbituric acid	405
4-Phenylazo-1-naphthol	410[b]
4-*o*-Hydroxyphenylazo-1-naphthol	420[c]
1-Phenylazo-2-naphthol	475[b]
1-*o*-Hydroxyphenylazo-2-naphthol	485[c]
1-Uracilyl-5′-azo-2-naphthol	480

[a] The Cary Recording Spectrophotometer was used with acetone as solvent.
[b] These values were obtained from Kuhn and Bär (10).
[c] These values were obtained from Foye and Jeffrey (11).

ing the formation, and decreased aqueous solubility. Quantitative analyses of the dried products indicated a ratio of one metal atom to one molecule of azo compound, as shown in Table III.

TABLE III.—ANALYSES OF SOME METALLIZED AZOPYRIMIDINES

Compound	Formula[a]	Analyses, %—Calcd.	Found[b]
1-Uracilyl-5′-azo-2-naphthol, Ni	$C_{14}H_{10}O_4N_4Ni$	C: 47.11	46.05
		H: 2.82	3.48
		Ni: 16.44	16.10
1-Uracilyl-5′-azo-2-naphthol, Co	$C_{14}H_{10}O_4Co.H_2O$	C: 44.81	45.85
		H: 3.22	3.30

[a] The chelates were dried *in vacuo* over $Mg(ClO_4)_2$ before analysis. Loss of weight was observed, so the products analyzed contained less than the theoretical amount of water of hydration.
[b] The carbon-hydrogen analyses were carried out at the Clark Microanalytical Laboratory, Urbana, Ill. The nickel analysis was done by the procedure of Hillebrand and Lundell (12), following decomposition of the chelate in 10% hydrochloric acid.

Biological Results.—Tests for anticancer activity carried out at the Southern Research Institute on several of the azopyrimidines did not reveal appreciable activity. These tests were carried out against Sarcoma 180 in mice, and a tumor weight reduction of 50% or more of the control was given a positive rating. The results of the tests are shown in Table IV.

EXPERIMENTAL

Preparation of Pyrimidines.—Uracil was obtained by the method of Wheeler and Merriam (13), and 6-methyluracil was obtained both by this procedure and that of Donleavy and Kise (14). 5-Aminouracil (uramil) was prepared according to the procedure of Hartman and Sheppard (15), while 5-amino-6-methyluracil was obtained by the procedure of Behrend and Osten (16).

Preparation of Azopyrimidines by Coupling into the Pyrimidine Ring. 5-*o*-Hydroxyphenylazobarbituric Acid.—The following preparation is representative of this method. *o*-Aminophenol (2.75 Gm., 0.025 mole) was dissolved in 7 ml. of concentrated hydrochloric acid and 14 ml. of water. The solution was cooled to 0–2° and diazotized by the rapid addition, with stirring, of a solution of 1.8 Gm. (0.025 mole) of sodium nitrite in 5 ml. of water. Barbituric acid (4.1 Gm., 0.032 mole) was dissolved in 600 ml. of water at room temperature, 3 ml. of 10% hydrochloric acid was added, and the cold solution of diazonium salt was added dropwise with stirring. After being stirred for one hour, the brown suspension was allowed to stand overnight. The product was collected, dried in a vacuum desiccator over calcium chloride, and extracted with Skellysolve B for forty-eight hours in a Soxhlet extractor. A yield of 4.4 Gm. (55%) of brown powder was obtained which decomposed with effervescence at 269–273°.

Preparation of Azopyrimidines by Coupling into the Naphthol Ring. 1-(6′-Methyluracilyl-5′-azo)-2-naphthol.—This preparation will be described to illustrate this method. 5-Amino-6-methyluracil (3.5 Gm., 0.025 mole) was suspended in 7 ml. of concentrated hydrochloric acid and 25 ml. of water. After being cooled to 0°, the mixture was treated with a solution of 2 Gm. (0.028 mole) of sodium nitrite in 5 ml. of water. Stirring was continued for forty minutes at 0–5°, and the pink solid was filtered, washed with water, and dried for one hour in a vacuum desiccator over calcium chloride. A yield of 4.7 Gm. (80% of theoretical) was obtained of diazo compound.

A solution of an equivalent of β-naphthol in 10% sodium hydroxide was added to a suspension of the diazonium salt in 100 ml. of water until no more diazonium salt was present, keeping the temperature below 5° and maintaining a pH of 8. After being stirred for ninety minutes, the red solid was filtered, resuspended in water, and brought to a pH

TABLE IV.—ANTICANCER ACTIVITY OF AZOPYRIMIDINES[a]

Azopyrimidine	Vehicle	Dose, mg./Kg. × No. Times Adm.	Mortality	Av. Mouse Wt. Change, Gm.	Tumor Wt., % of Control
1-Uracilyl-5′-azo-2-naphthol	CMC	500 × 4	0/10	+1.2	57
		200 × 4	0/10	+4.1	54
5-*o*-Hydroxyphenylazouracil	CMC	200 × 4	0/10	+4.2	75
5-*o*-Hydroxyphenylazo-6-methyluracil	CMC	250 × 4	0/10	+1.3	56
		200 × 4	1/10	+3.5	66
5-*o*-Hydroxyphenylazo-2-thio-6-methyluracil	CMC	200 × 4	1/10	+2.3	46
		150 × 4	3/10	+1.0	60
5-Phenylazobarbituric acid	CMC	250 × 4	0/10	+0.2	76
		200 × 4	1/10	+2.1	66

[a] Determined at the Southern Research Institute, Birmingham, Ala. Results were made available by Dr. R. W. Brockman.

of 6 with hydrochloric acid. The bright pink product, m. p. 120–145°, was filtered, washed with water and ether, extracted with water in a Soxhlet extractor for seventy-two hours. The resulting scarlet solid decomposed at 273–275°.

Preparation of Azopyrimidines by Ring Closure. Ethyl *o* - Hydroxyphenylazoacetoacetate.—To a solution of sodium (2.3 Gm., 0.1 mole) in 40 ml. of absolute ethanol contained in a 500-ml. flask protected from moisture was added 13 Gm. (0.1 mole) of ethyl acetoacetate dropwise with stirring. A solution of diazotized *o*-aminophenol (0.1 mole) in 65 ml. of 10% hydrochloric acid was added gradually with stirring at 0–5°, and the resulting yellow-brown suspension was stirred for two hours and allowed to stand overnight. Water was then added, and the solid was filtered, washed with water, and dried *in vacuo*. The product was purified by solution in hot absolute ethanol and precipitation with water. A yield of 51% of yellow product was obtained which melted at 160.5–162°.

Anal.—Calcd. for $C_{12}H_{14}O_4N_2$: C, 57.59; H, 5.64. Found: C, 57.56; H, 5.83.

5 - *o* - Hydroxyphenylazo - 6 - methyluracil.—A solution of 2.3 Gm. (0.1 mole) of sodium in 75 ml. absolute ethanol contained in a 500-ml. flask protected from moisture was treated with 3 Gm. (0.05 mole) of dried urea, which dissolved with stirring, and 12.5 Gm (0.05 mole) of ethyl *o*-hydroxyphenylazoacetoacetate. The reaction mixture was refluxed for three and one-half hours and allowed to stand overnight. The alcohol was removed under reduced pressure, and the dark red residue was dissolved in a minimum (40 ml.) of hot water and acidified with glacial acetic acid. An oil resulted which solidified after chilling, and the solid was collected, dried *in vacuo*, and pulverized to yield 5.4 Gm. (44% yield) of a dark red powder. The product was purified by dissolving in alkaline solution and precipitating with acid, drying, and extracting with Skellysolve B for two days. After vacuum drying, the product decomposed with effervescence at 194°.

In the corresponding reaction with thiourea, it was necessary to use twice as much ethanol to facilitate stirring.

Metallization Reactions.—The reaction of 1-uracilyl-5′-azo-2-naphthol with nickel ion will be described as example. The azo compound (1.4 Gm., 0.005 mole) was dissolved in 40 ml. of triethanolamine and 20 ml. of water with stirring at steam-bath temperature. A solution of 1.0 Gm. (0.0033 mole) of nickel nitrate was added during one hour with stirring at 85–90°, and the stirring was continued for two and one-half hours at this temperature. At the end of this time, a paper chromatogram indicated the reaction to be complete by the presence of a single band. An excess of water was added, the mixture was heated to 85°, and the product was filtered while hot. After drying *in vacuo*, it was pulverized and extracted in a Soxhlet extractor first with water and then with acetone. A yield of 0.55 Gm. (32%) of dark red solid was obtained.

SUMMARY

1. *o*-Hydroxyazo derivatives of uracil and substituted uracils have been prepared by both coupling and ring closure procedures. Chemical and spectral evidence indicates that they exist mainly as quinone hydrazones.

2. The *o*-hydroxyazopyrimidines were found to chelate heavy metal ions at elevated temperatures giving products having a 1:1 ratio of metal to azo compound.

3. The *o*-hydroxyazopyrimidines did not show any significant anticancer activity when tested in mice against Sarcoma 180.

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The Influence of Bile Salts on Glycoside Biosynthesis in *Digitalis purpurea* I.*

Sodium Cholate

By FRANK T. CHAN and DANIEL P. N. TSAO†

Concentrations of 0.02, 0.10, 0.50, and 2.50% sodium cholate were injected into digitalis plants growing in pots under controlled greenhouse conditions. Sodium cholate in the concentrations of 0.50 and 2.50% produced marked inhibition of total glycoside formation. A periodic assay of total glycoside biosynthesis following injections with 0.50% sodium cholate injection indicated that the treated digitalis plants produced less glycosides than the controls.

IN THE LAST DECADE many reports have been made on the growth of plants following the use of steroids as growth regulators. Controversial reports have been made on the influence of sex hormones on the growth of such plants (1, 2). There have been several reports about the inhibiting effect of sodium cholate on plant metabolism (3–6). Deysson and Deysson (3, 4) reported that sodium cholate in concentrations between 0.01 and 0.25% exerted a marked toxic effect on meristematic cells of *Allium cepa*. Deysson and Charbonnier (5, 6) produced a retardation of both growth and mitotic activity of *Allium cepa* by treatment with concentration of 0.05% of sodium cholate. Much, *et al.* (7), found that a cholesterol mixture increased the growth of digitalis. A similar report by Tsao and Youngken (8) indicated that 0.5 and 2.0% cholesterol in suspension produced an increased glycoside production in digitalis plants grown in hydroponic media. However, sodium cholate, a bile salt possessing a basic structure similar to cholesterol and being more soluble in water than the latter, was employed in this study.

This study deals with the influence of various concentrations of sodium cholate on glycoside biosynthesis and with the periodic investigation of the effect of sodium cholate on glycoside formation in this plant.

MATERIALS AND METHODS

Digitalis plants employed in this study were developed under carefully controlled greenhouse conditions.[1] All seedlings and mature plants were grown from the strain of *Digitalis purpurea* Linné which produced flowers of mixed colors, i. e., purple, white, lavender. Seeds were germinated in the greenhouse in culture flats containing soil composed of 2 parts of compost and 1 part of peat moss. The seedlings were transplanted into pots containing the same soil mixture.

These plants were subjected to artificial illumination for 12 hours daily for one month prior to the beginning of injections. The light was supplied by white 40-watt Sylvania fluorescent lamps, which were suspended 7 to 9 inches above the plants. Growth measurements of all plants were recorded daily during the experimental period. The two largest leaves were selected from each plant for measurement. The length and width of the blade and the length of petiole and blade were recorded.

Sodium cholate solutions and distilled water were adjusted to a pH of 6.8 by 10% sodium hydroxide or 10% hydrochloric acid. The solutions were administered into the plants by injection apparatus (Fig. 1). The outlet of a 10-ml. syringe was fitted with a No. 21-gauge needle by means of intravenous rubber tubing. The air bubbles were removed from the injection apparatus and a volume of specified solutions was injected into the petiole at a speed of about 1 ml. per hour exercising extreme care in order to avoid overinjury of the area of contact. The pressure applied to the plunger was supplied by 2 or 3 rubber bands ($^1/_4$ x $3^3/_4$″). The plunger was adjusted occasionally during the injection period so that the required speed could be maintained.

At the end of the treatment the plants were harvested and the weight of fresh leaves of each group was recorded. The fresh materials were then transferred immediately into a hot air circulating dryer and dried at a temperature between 118 and 120 degrees F. Dried weight and moisture content were measured. The dried leaves were ground into a No. 60 powder and stored in an air-tight container for glycoside analyses.

Digitalis tinctures were prepared according to U. S. P. XV method (9). The total glycoside was assayed by using a modified Knudson-Dresbach method (10). The absorbance was compared at

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† Present address: Pharmacognosy Department, School of Pharmacy, Oregon State College, Corvallis.

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525 mμ in a 1-cm. cell by the use of a Beckman Model DU Photoelectric Quartz Spectrophotometer. A standard curve was constructed from a tincture prepared from U. S. P. Digitalis Reference Standard. Unknown tinctures were then compared with standard digitalis reference tincture and the glycoside yields were expressed in per cent potency of the latter reference standard, expressed as 100%.

EXPERIMENTAL

Part I.

The Influence of Various Concentrations of Sodium Cholate on the Glycoside Biosynthesis.—Seventy digitalis plants were transplanted into pots on September 8, 1955, and were grown under artificial illumination for one month before the experimental study. These plants were 151 days old from the date of germination and were divided into 7 groups; each group consisted of 10 plants. Two groups were designated as controls or untreated, i. e., groups A and B. The other five groups (groups C to G) were treated with specified solutions such as distilled water; 0.02, 0.10, 0.50, and 2.50 per cent of sodium cholate. Each plant of the treated groups received 5 ml. of the respective solutions at a rate of about 1 ml. per hour by injecting into the petiole. One such injection was given each day for three successive days. The first injection was started on November 30, 1955.

The plants in Group G, injected with 2.50% of sodium cholate, showed severe toxicity, such as wilting and chlorosis, after the second injection. Plants in this group were harvested after the second injection with the control, Group B. The remaining 5 groups (A, C, D, E, F) were harvested on December 3, 1955. The plant material was dried and assayed for total glycosides. Results are shown in Tables I and II and Fig. 2.

TABLE I.—ARRANGEMENT OF DIGITALIS PLANTS DURING EXPERIMENTAL FEEDING WITH SODIUM CHOLATE

Part	Group	Experiment	Plants		Injection				Leaf Yield		Moisture
			Age, Days	No.	pH of Soln.	Volume (ml.)	Rate (hr.)	No. of Inject.	Fresh (Gm./Group)	Dry	(per cent)
I	A	Control	151	10	..	..	..	..	220.0	27.3	87.60
	B	Control	151	10	..	..	..	..	210.0	25.8	87.71
	C	Dist. water	151	10	6.8	5.0	*ca.* 5	3	238.0	27.0	88.66
	D	Sod. cholate 0.02%	151	10	6.8	5.0	*ca.* 5	3	218.0	24.6	88.74
	E	Sod. cholate 0.10%	151	10	6.8	5.0	*ca.* 5	3	202.0	23.1	88.05
	F	Sod. cholate 0.50%	151	10	6.8	5.0	*ca.* 5	3	225.0	28.8	87.21
	G	Sod. cholate 2.50%	151	10	6.8	5.0	*ca.* 5	2	125.0	24.1	80.69[a]
II	1	Dist. water	189	6	6.8	5.0	*ca.* 5	1	170.0	18.0	89.41
	2	Sod. cholate 0.50%	189	6	6.8	5.0	*ca.* 5	1	146.0	16.0	89.04
	3	Dist. water	190	6	6.8	5.0	*ca.* 5	2	150.0	16.0	89.33
	4	Sod. cholate 0.50%	190	6	6.8	5.0	*ca.* 5	2	106.0	13.0	87.73
	5	Dist. water	191	6	6.8	5.0	*ca.* 5	3	154.0	17.0	88.98
	6	Sod. cholate 0.50%	191	6	6.8	5.0	*ca.* 5	3	139.0	17.0	87.77
	7	Dist. water	192	6	6.8	5.0	*ca.* 5	4	173.0	19.0	89.02
	8	Sod. cholate 0.50%	192	6	6.8	5.0	*ca.* 5	4	148.0	19.0	87.18
	9	Dist. water	193	6	6.8	5.0	*ca.* 5	5	150.0	17.0	88.67
	10	Sod. cholate 0.50%	193	6	6.8	5.0	*ca.* 5	5	153.0	19.0	87.57

[a] Plants showed severe wilting and chlorosis.

TABLE II.—GLYCOSIDE YIELDS OF DIGITALIS PLANTS TREATED WITH VARIOUS CONCENTRATIONS OF SODIUM CHOLATE BY THE INJECTION METHOD

Part	Group	Experiment	Activity[a] (Per Cent)	Per Cent Difference
I	A	Control	160.0	
	B	Control	161.0	
	C	Dist. water	154.0	− 3.8 (*vs.* Control A)
	D	Sod. cholate 0.02% (0.00005 *M*)	153.5	− 4.1 (*vs.* Control A) − 0.3 (*vs.* Dist. water)
	E	Sod. cholate 0.10% (0.00023 *M*)	140.0	−12.5 (*vs.* Control A) − 9.1 (*vs.* Dist. water)
	F	Sod. cholate 0.50% (0.00116 *M*)	129.5	−19.1 (*vs.* Control A) −16.0 (*vs.* Dist. water)
	G	Sod. cholate 2.50% (0.00581 *M*)	114.5	−28.9 (*vs.* Control B)
II	1	Dist. water	116.5	
	2	Sod. cholate 0.50% (0.00116 *M*)	106.5	−8.6
	3	Dist. water	110.5	
	4	Sod. cholate 0.50% (0.00116 *M*)	105.0	−5.0
	5	Dist. water	118.0	
	6	Sod. cholate 0.50% (0.00116 *M*)	109.8	−7.0
	7	Dist. water	113.0	
	8	Sod. cholate 0.50% (0.00116 *M*)	104.5	−7.5
	9	Dist. water	120.0	
	10	Sod. cholate 0.50% (0.00116 *M*)	110.5	−7.9

[a] In comparison with Digitalis U. S. P. Reference Standard which was expressed as 100%.

This study indicated that sodium cholate in concentrations of 0.10, 0.50, and 2.50 per cent inhibited the glycoside biosynthesis in digitalis by 12.5, 19.1, and 28.9 per cent respectively as compared with the controls, and the concentrations of 0.10 and 0.50 per cent showed a decrease in glycoside content of 9.1 and 16.0 per cent respectively as compared with the plants receiving the injection of distilled water (Table II and Fig. 2). The lowest concentration of sodium cholate, 0.02%, did not show significant inhibition on glycoside biosynthesis. There was no significant growth difference between treated and untreated groups except that the plants injected with 2.50% sodium cholate showed severe wilting and chlorosis.

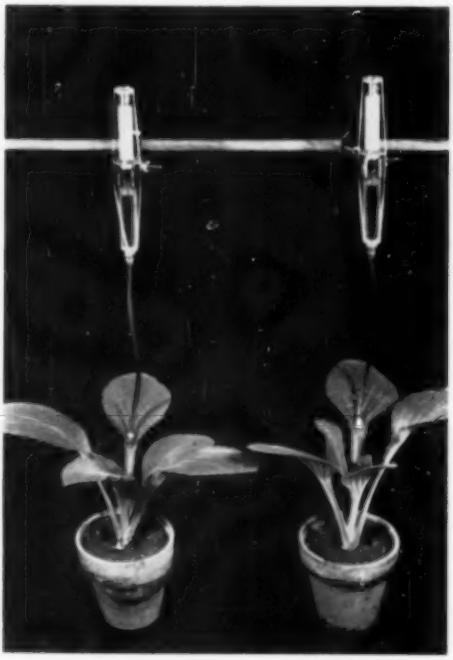

Fig. 1.—Injection apparatus for the administration of sodium cholate in digitalis plants.

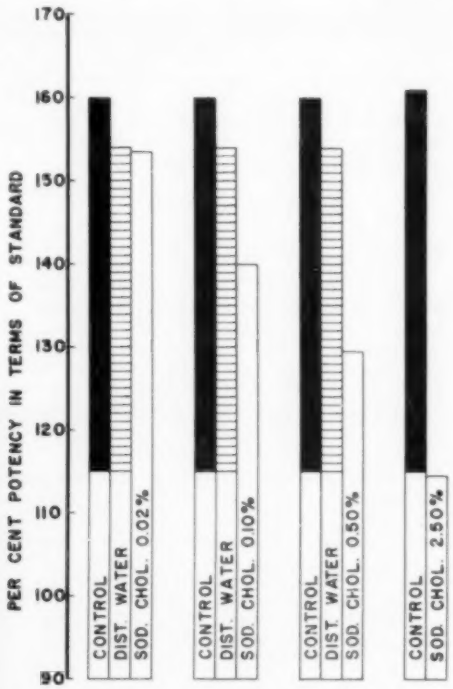

Fig. 2.—Analysis of total glycosides of digitalis feedings with sodium cholate.

Part II.

Periodic Study of the Effect of Sodium Cholate on the Glycoside Biosynthesis.—Since 0.10, 0.50, and 2.50 per cent sodium cholate solutions inhibited the biosynthesis of digitalis glycosides, it was deemed important to further study the periodic effect of sodium cholate on the glycoside, formation. Sixty digitalis plants which had been grown in the cold frame since September 8, 1955, were transplanted into pots on December 7, 1955, and were allowed to grow in the greenhouse under artificial illumination for one month before the periodic study. These plants were 189 days old and were divided into 10 groups; each group consisted of 6 plants. Five groups were designated as controls (Groups 1, 3, 5, 7, 9) and the other five groups were assigned as experimentals (Groups 2, 4, 6, 8, 10). Each treated group had its own control group, according to their order of increasing numbers, such as Groups 1 and 2; 3 and 4; etc. The control groups were treated with distilled water while the experimental groups were treated with 0.50% sodium cholate.

Each plant received 5 ml. of the respective solution at a rate of about 1 ml. per hour by the injection method. The injection commenced on January

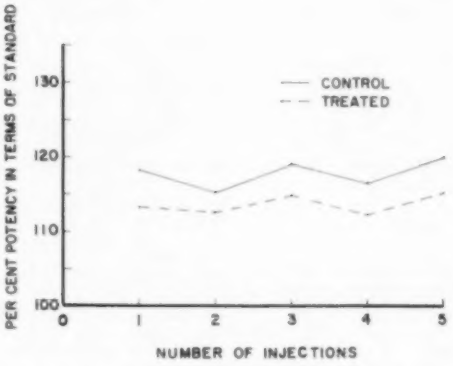

Fig. 3.—Periodic study of glycoside formation in digitalis following injections of 0.5% sodium cholate (one to five day intervals).

11, 1956. The plants in these 10 groups were injected with their respective solutions and on the second day after the first injection, the plants of Groups 1 and 2 were harvested while the remaining 8 groups were given a second injection. On the third day, Groups 3 and 4 were harvested while the remaining groups received the third injection. The injections and harvests were continued in the same manner until Groups 9 and 10 were collected on January 16, 1956. The plant material was weighed, dried, and assayed for the total glycosides. Results are shown in Tables I and II and Fig. 3.

This periodic study of 0.50 per cent sodium cholate on digitalis plants showed a decrease in glycoside content as compared with the controls by 5.0 to 8.6 per cent, but the difference was not significant compared with the results obtained in the previous study in Part I (Table II and Figs. 2 and 3).

DISCUSSION

The data on total glycoside yield in Part II revealed that 0.50% sodium cholate inhibited glycoside formation by 5.0 to 8.6 per cent in comparison with those plants treated with distilled water, while the same concentration in Part I produced an inhibition of glycoside production by 16.0% in comparison with plants treated with distilled water (Table II). The variance of results on glycoside inhibition may be attributed to such factors as age of digitalis plants, temperature, and season. The plants used in Part I were young (151 days old) and were grown in the greenhouse all of the time while those employed in Part II were firstly, older (189 days old) and secondly, were grown in the cold frame for 62 days (from September 8, 1955 to December 9, 1955). Tsao and Youngken (11) indicated that the age of plants and season of growth have a great influence on the glycoside formation. It is, therefore, concluded that the variance in results on glycoside inhibition in Parts I and II can be attributed to the age of plant and the environmental conditions which interfere with the anabolism of the digitalis plants in the production of glycosides.

The possibility of physical factors, like osmotic pressure, which could exert a marked inhibition or toxic symptoms has been eliminated in this study. The osmotic pressure of 2.50 per cent (0.00116 *M*) sodium cholate, if it were completely dissociated, would be less than the osmotic pressure required to rupture plant cells. Tsao and Youngken (8) indicated that 0.04 *M* potassium chloride in Hoagland solution did not show any inhibition effect on glycoside formation in digitalis plants. Since digitalis plants, injected with distilled water, showed no significant difference in glycoside formation from the untreated plants, hypotonicity was eliminated.

Various workers (3–6, 12–15) showed the inhibiting effect of bile salts on plants and animals. The reports by Deysson and Deysson (3, 4) and Deysson and Charbonnier (5, 6) indicated that sodium cholate had a toxic effect on the meristematic cells, and the retardation of both growth and mitotic activity in *Allium cepa*. The plants in Group G, injected with 2.50% sodium cholate solution, showed severe toxic symptoms as evidenced by wilting and chlorosis. Wang (12) and Pihar (13) showed that bile salts inhibited succinic oxidase. Kiese and Reinwein (14) reported a depression of oxygen uptake in heart muscle after treating with sodium cholate solution. Manni (15) showed that bile salts inhibited the adenosine triphosphate (ATPase) activity of myosin. It is concluded that the inhibition of glycoside formation by sodium cholate in digitalis may be attributed, partially or wholly, to the above factors.

SUMMARY

A study has been made on the influence of sodium cholate on the glycoside biosynthesis in *Digitalis purpurea*. The following salient data were observed:

1. A modification has been described for introducing chemical compounds into digitalis leaf tissues.

2. Concentrations of 0.02, 0.10, 0.50, and 2.50 per cent sodium cholate were injected into digitalis plants growing in pots under controlled greenhouse conditions. Sodium cholate in the concentrations of 0.10, 0.50, and 2.50 per cent produced marked inhibition of total glycoside formation.

3. A periodic assay of total glycoside biosynthesis following injections with 0.50 per cent sodium cholate indicated that the treated digitalis plants produced less glycosides than the controls.

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The Influence of Bile Salts on Glycoside Biosynthesis in *Digitalis purpurea* II.*

Sodium Deoxycholate, Dehydrocholate, Glycocholate, and Taurocholate

By DANIEL P. N. TSAO†

Concentrations of 0.10, 0.25, 1.50, and 5.00% sodium glycocholate were administered into digitalis plants by injection. Sodium glycocholate in the concentration of 0.25% stimulated the glycoside formation while 1.50% sodium glycocholate showed no significant effect on glycoside production and 5.00% sodium glycocholate inhibited glycoside production significantly. Sodium deoxycholate, dehydrocholate, and taurocholate each in the concentration of 0.25% showed no significant effect on the biosynthesis of the total glycosides.

REPORTS IN THE LITERATURE have indicated that bile salts influence the plant metabolism. Clark (1) abolished heteroauxin transport in *Avena coleoptiles* with a concentration of 10 p. p. m. of sodium glycocholate. Lewis (2) in the study of transpiration of *Ficus elastica* showed that the hydrophobic nature or the surface of the wall was destroyed by concentrations of 10 and 50 p. p. m. of sodium glycocholate. Chan and Tsao (3) indicated that sodium cholate in concentrations of 0.50 and 2.50% produced a marked inhibition on glycoside biosynthesis in *Digitalis purpurea*. Deysson (4) reported that sodium taurocholate in nonpoisoning doses did not increase permeability in plant cells of *Allium cepa* bulbs, *Elodea canadensis* leaves, and *Pisum* seedlings. However, Dimaggio (5, 6) found that sodium taurocholate inhibited germination and development of wheat seedlings.

In so far as glycoside biosynthesis in digitalis was inhibited by sodium cholate, it is important to investigate other bile salts having the same effect on this plant. This study deals with the effects of high titers of sodium deoxycholate, dehydrocholate, glycocholate, and taurocholate on glycoside biosynthesis in *Digitalis purpurea*.

MATERIALS AND METHODS

Digitalis purpurea plants of approximately the same size and age were grown in the greenhouse according to the method previously described by Chan and Tsao (3). Chemically pure sodium deoxycholate, dehydrocholate, glycocholate, and taurocholate were dissolved in distilled water and solutions were prepared and adjusted to a pH of 6.3. They were administered into the petiole of the plant by injection apparatus (3) at a rate of about one ml. per hour.

Representative leaves were selected during the experimental period for the study of growth measurements and leaf size, and rates of growth were compared with those of controls.

Tinctures were made from dried and powdered leaves according to the U. S. P. XV procedure (7). These were analyzed for total glycosides by a modified Knudson-Dresbach colorimetric method (3) and spot comparisons were made with a modified U. S. P. XV biological procedure (7), during which pigeons weighing from 400 to 600 grams were used. All material was compared with that of a U. S. P. Reference Digitalis Powder.

EXPERIMENTAL

Thirty digitalis plants were transplanted into pots on November 15, 1954 under carefully controlled greenhouse conditions.[1] They were divided into five groups (Groups A–E). Each group consisted of six plants. Group A was designated as control. The remaining four groups (B–E) were treated with 0.25% solutions of sodium deoxycholate, dehydrocholate, glycocholate, and taurocholate respectively. Each plant of the treated groups received five ml. of the respective solutions at a rate of about one ml. per hour by injection into the petiole. Three injections were given to each plant of the treated groups. Each injection was administered at interval of fourteen days. The first injection was given on December 28, 1954. The leaves were harvested on February 23, 1955. The growth measurements were recorded during the experimental period. There was no significant difference in growth between plants in treated and control groups. Tinctures were prepared from dried and powdered leaves and assayed for total glycosides. Results are shown in Tables I and II and Fig. 1. Plants treated with sodium deoxycholate, dehydrocholate, and taurocholate (Groups B, C, E), each in the concentration of 0.25%, were 6.3, 10.0, and 11.1 per cent

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† Present address: Pharmacognosy Department, School of Pharmacy, Oregon State College, Corvallis.

[1] The author wishes to acknowledge the generous assistance given during the growth of digitalis plants and the injection procedure to Drug Plant Laboratories, College of Pharmacy, University of Washington, Seattle.

TABLE I.—ARRANGEMENT OF DIGITALIS PLANTS DURING EXPERIMENTAL INJECTION WITH BILE SALTS

Group	Experiment	No. of Plants	Injection: pH of Soln.	Injection: Volume (ml.)	Injection: Rate (hr.)	Injection: No. of Inject.	Leaf Yield Fresh (Gm./group)	Leaf Yield Dry (Gm./group)	Moisture, %
A	Control	6	..	..	..	..	70.0	17.0	76
B	Sod. deoxycholate 0.25%	6	6.3	5	*ca.* 5	3	70.2	19.3	73
C	Sod. dehydrocholate 0.25%	6	6.3	5	*ca.* 5	3	79.2	17.3	78
D	Sod. glycocholate 0.25%	6	6.3	5	*ca.* 5	3	83.4	21.6	74
E	Sod. taurocholate 0.25%	6	6.3	5	*ca.* 5	3	91.0	23.1	76
F	Control	6	..	..	..	..	70.3	10.1	86
G	Control	4	..	..	..	..	44.0	5.6	87
H	Sod. glycocholate 0.10%	6	6.3	5	*ca.* 5	5	91.9	13.3	86
I	Sod. glycocholate 1.50%	6	6.3	5	*ca.* 5	2	52.4	9.6	82
J	Sod. glycocholate 5.00%	6	6.3	5	*ca.* 5	1	54.0	10.5	81
K	Glycine 0.25%	6	6.3	5	*ca.* 5	5	65.1	9.6	85

TABLE II.—RESULTS OF GLYCOSIDE ANALYSIS FOLLOWING BILE SALTS FEEDINGS OF *Digitalis purpurea* BY INJECTION METHOD

Group	Experiment	Colorimetric Assay: Potency,[a] %	Colorimetric Assay: Difference, %	No. of Pigeons	Biological Assay: Potency[a] %	Biological Assay: Difference, %
A	Control	125.0		6	90.5 ± 3.3	
B	Sod. deoxycholate 0.25%	116.7	− 6.3			
C	Sod. dehydrocholate 0.25%	112.5	−10.0			
D	Sod. glycocholate 0.25%	161.7	+29.4	6	120.0 ± 5.8	+32.6 (*vs.* A)
E	Sod. taurocholate 0.25%	111.2	−11.1			
F	Control	162.0		6	101.3 ± 5.3	
G	Control	166.0		6	98.4 ± 4.9	
H	Sod. glycocholate 0.10%	178.0	+ 9.9 (*vs.* F)	6	105.6 ± 6.8	+ 4.2 (*vs.* F)
I	Sod. glycocholate 1.50%	170.0	+ 5.0 (*vs.* F)	6	102.9 ± 7.1	+ 1.5 (*vs.* F)
J	Sod. glycocholate 5.00%	132.0	−20.5 (*vs.* G)	6	82.7 ± 6.0	−16.0 (*vs.* G)
K	Glycine 0.25%	184.0	+13.5 (*vs.* F)	6	110.5 ± 6.3	+ 9.1 (*vs.* F)

[a] In comparison with digitalis standard which was expressed as 100 per cent.

lower in total glycosides than the control, respectively, while digitalis plants treated with 0.25% sodium glycocholate showed 29.4% by colorimetric assay and 32.6% by biological assay higher in glycoside production than the control. (Table II and Fig. 1.)

On June 10, 1955, thirty-four digitalis plants were transplanted into pots in the greenhouse. They were divided into six groups (Groups F–K). Group G consisted of four plants while each of the others (F, H–K) consisted of six plants. Two groups (F, G) were used as control groups.

The other four groups (H–K) were treated with the solutions of the following chemicals; 0.1, 1.5, and 5.0 per cent of sodium glycocholate for groups H, I, and J, respectively, and 0.25 per cent glycine for group K. Each plant in the treated groups received one injection of its specified solution every other day. The injection was started on July 21, 1955. Each of the treated groups given five injections, except the plants in Group J (5% sodium glycocholate), showed severe wilting and chlorosis after the first injection and those in Group I (1.5% sodium glycocholate) developed wilting to some extent after the second injection. The plants in Group J were harvested on the third day after the first injection with those plants in Group G as control. The growth measurements were recorded during the injection period. There was no significant difference in growth rate between plants in treated and control groups except those plants receiving 1.5 and 5.0 per cent sodium glycocholate showed wilting and chlorosis. Plants in Groups F, H, I, and J were harvested on July 31, 1955. They were dried and ground into a No. sixty powder and stored in air-tight containers for both chemical and biological assays. Results are shown in Tables I and II and Fig. 1. The potency of digitalis leaves treated with 0.1 and 1.5 per cent sodium glycocholate were 9.9 and 5.0 per cent by colorimetric assay and 4.2 and 1.5 per cent by bio-assay higher than the control, while that treated with 5.0 per cent sodium glycocholate was 20.5 per cent by colorimetric assay and 16.0 per cent by bio-assay lower than the control. Plants treated with 0.25 per cent glycine showed 13.5 per cent by colorimetric method and

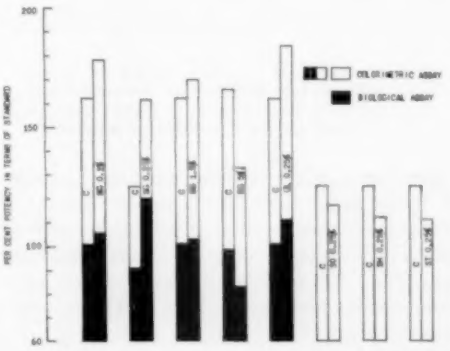

Fig. 1—Analysis of total glycosides of digitalis feedings with bile salts and glycine. C, control; SG, sodium glycocholate; GL, glycine; SO, sodium deoxycholate; SH, sodium dehydrocholate; ST, sodium taurocholate.

9.1 per cent by biological assay higher than the control. (Table II and Fig. 1).

DISCUSSION

Sodium deoxycholate, dehydrocholate, and taurocholate each in the concentration of 0.25 per cent inhibited glycoside formation in *Digitalis purpurea* by 6.3, 10.0, and 11.1 per cent (Table II and Fig. 1). The degree of inhibition in these cases is not very significant because biological variance accounts for ±10.0 per cent. On the other hand, sodium glycocholate showed both stimulating and inhibiting effects on glycoside biosynthesis with different concentrations. The lower concentration (0.1 per cent sodium glycocholate) showed no significant effect on glycoside biosynthesis; the medium concentration (0.25 per cent) stimulated the glycoside formation by 29.4 and 32.6 per cent; the medium high concentration (1.5 per cent) produced no significant effect on total glycosides; and the highest concentration (5.0 per cent) inhibited glycoside biogenesis by 20.5 and 16.0 per cent. (Table II and Fig. 1). Clark (1) indicated that sodium glycocholate 10 p. p. m. abolished heteroauxin transport in *Avena coleoptiles*. Lewis showed that the hydrophobic nature or the surface of the wall in *Ficus elastica* was destroyed by concentrations of 10 and 50 p. p. m. of sodium glycocholate. It might be possible that sodium glycocholate acts as a growth sensitizing factor for the digitalis plant to enhance anabolic metabolism so that the production of glycosides is increased. Although sodium glycocholate contains the glycine radical ($NHCH_2COOH$), the stimulation effect of glycocholate could not be entirely accredited to the glycine portion, because 0.25 per cent glycine stimulated the glycoside formation by 13.5 and 9.1 per cent and 0.25 per cent sodium glycocholate increased glycoside production by 29.4 and 32.6 per cent.

SUMMARY

The influence of sodium deoxycholate, dehydrocholate, glycocholate, and taurocholate on glycoside biosynthesis in *Digitalis purpurea* has been studied.

1. Concentrations of 0.10, 0.25, 1.50, and 5.00 per cent sodium glycocholate were injected into digitalis plants growing under controlled greenhouse conditions. Sodium glycocholate in concentrations of 0.10 and 1.50 per cent showed no significant effect on total glycoside formation; 0.25 per cent sodium glycocholate stimulated glycoside biosynthesis; and 5.00 per cent sodium glycocholate inhibited total glycoside production.

2. Sodium deoxycholate, dehydrocholate, and taurocholate, each in the concentration of 0.25 per cent, showed no significant inhibition on the biosynthesis of the total glycoside.

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Preparation of New Derivatives of Anisic Acid*

By SEYMOUR LIBERMAN and FRANK W. BOPE

The synthesis of five new esters of anisic acid with amino alcohols is discussed and their analyses are presented. Pharmacological tests indicate local anesthetic properties.

A NUMBER OF alkoxy substituted benzoic acid derivatives have been esterified with alkyl substituted amino alchohols known to possess analgetic properties such as diethylaminoethanol (1). The resulting alkoxybenzoates have been studied pharmacologically and show local anesthetic activity (2, 3, 4).

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Based in part upon a thesis submitted by Seymour Liberman to the Graduate School of The Ohio State University in partial fulfillment of the requirements for the degree of Master of Science.

The work undertaken by the authors was the preparation of some new esters of anisic acid in an attempt to produce some new local anesthetics. Five new esters were prepared from substituted amino alcohols and the acid chloride of anisic acid, 4-methoxybenzoyl chloride.

EXPERIMENTAL

Preparation of 2-Dimethylamino-1-methylethyl Anisate Hydrochloride and 2-Di-n-butylaminoethyl Anisate Hydrochloride.—The preparation of 2-dimethylamino-1-methylethyl anisate hydrochloride illustrates the method employed. One-tenth mole (17.1 Gm.) of anisoyl chloride was added in divided portions, with stirring, to a solution of 0.1 mole (10.3 Gm.) of 1-dimethylamino-2-propanol in

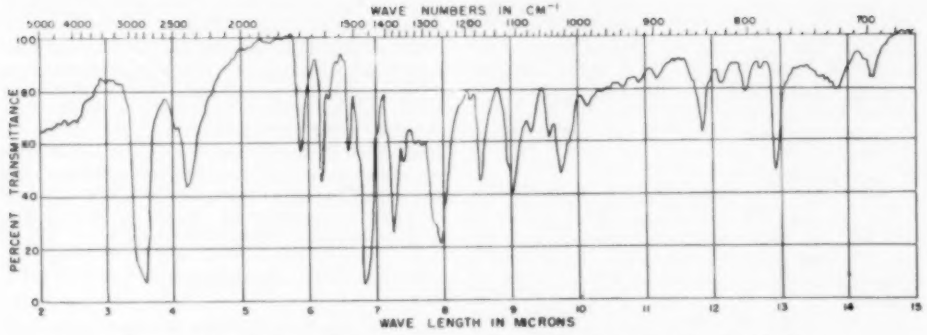

Fig. 1.—Infrared absorption spectrograph of 2-anilinoethyl anisate hydrochloride.

100 ml. of anhydrous, thiophene-free benzene. The reaction mixture was mechanically stirred for four hours and allowed to stand overnight (60 hours was required for the 2-di-*n*-butylaminoethyl ester). The resulting precipitate was filtered, dissolved in a minimum amount of hot acetone, and crystallized from the cooled solvent. Upon further recrystallization and drying in an Abderhalden pistol at 100° and 2 mm. pressure for six hours over phosphorus pentoxide, the crystals melted at 167°. The yield was 17.5 Gm., corresponding to 63.9% of the theoretical value. The 2-di-*n*-butylaminoethyl anisate hydrochloride melted at 109°. The yield of 20.5 Gm. was 59.8% of the theoretical value.

Anal.—Calcd. for $C_{13}H_{19}NO_3.HCl$: C, 57.03; H, 7.36; mol. wt. 274. Found: C, 56.66; H, 7.10; mol. wt. 287.

Anal.—Calcd. for $C_{18}H_{29}NO_3.HCl$: C, 62.87; H, 8.50; mol. wt. 344. Found: C, 63.09; H, 8.80; mol. wt. 345.

Preparation of 2-Isopropylaminoethyl Anisate Hydrochloride.—An equimolar portion of anisoyl chloride (17.1 Gm.) was added to 0.1 mole (10.3 Gm.) of 2-isopropylaminoethanol in 100 ml. benzene. The mixture was mechanically stirred for three hours and allowed to stand overnight (ten hours). The entire reaction mixture was made alkaline with 0.1 *N* sodium hydroxide solution and the separated benzene layer was washed with distilled water and dried over anhydrous sodium sulfate. Hydrogen chloride gas was bubbled in directly forming the ester hydrochloride. The excess benzene was removed under reduced pressure and the product was crystallized from acetone. This compound was dried in the manner described above; its melting point was 183-184° and the yield of 1.5 Gm. was 5.5% of the calculated amount.

Anal.—Calcd. for $C_{13}H_{19}NO_3.HCl$: C, 57.03; H, 7.36; mol. wt. 274. Found: C, 57.71; H, 7.34; mol. wt. 270.

Preparation of 2-Diethylamino-1-methylethyl Anisate Hydrochloride.—Ten milliliters of absolute alcohol, saturated with hydrogen chloride gas, was added to 25 ml. of anhydrous benzene. One-tenth mole (13.1 Gm.) of 1-diethylamino-2-propanol was added followed by an equivalent amount of anisoyl chloride (17.1 Gm.) dissolved in 75 ml. of benzene. The subsequent procedure was identical with that utilized in the preparation of 2-isopropylaminoethyl anisate hydrochloride. The melting point of the product was 127° and the yield was 5.3 Gm. or 17.6% of the theoretical amount.

Anal.—Calcd. for $C_{15}H_{23}NO_3.HCl$: C, 59.69; H, 8.02; mol. wt. 302. Found: C, 59.41; H, 7.38; mol. wt. 318.

Preparation of 2-Anilinoethyl Anisate Hydrochloride.—This product was prepared by addition of a hydrogen chloride catalyst to a mixture of anisoyl chloride and 2-anilinoethanol in anhydrous benzene. Hydrogen chloride was bubbled in at this point to

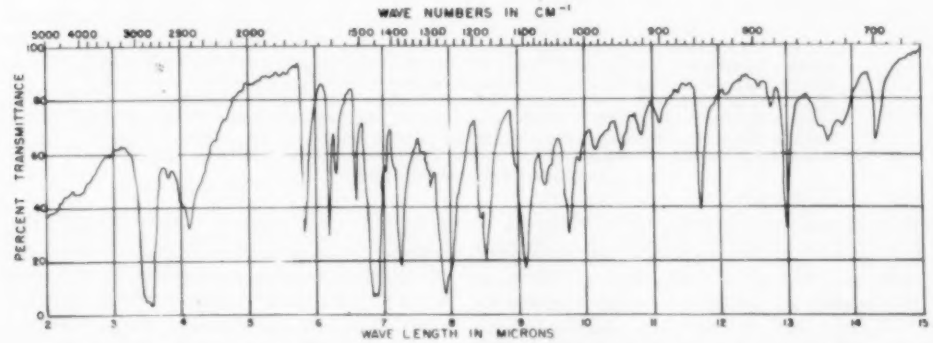

Fig. 2.—Infrared absorption spectrograph of 2-di-*n*-butylaminoethyl anisate hydrochloride.

preferentially aid in the formation of the ester rather than the amide (5). The mixture was refluxed for four hours and the benzene removed by distillation. The remaining mass was refrigerated overnight at 4°, washed with cold benzene, and crystallized from acetone. This compound was dried similarly to the other esters; the melting point was 133° and the resultant yield was 9.3 Gm. or 30.2% of the theoretical yield.

Anal.—Calcd. for $C_{16}H_{17}NO_3.HCl$: C, 62.44; H, 5.90; mol. wt. 308. Found: C, 60.13; H, 7.90; mol. wt. 314. Because of the discrepancies between the calculated values for the carbon and hydrogen content and those actually found, this compound is reported to be of doubtful compositon. The molecular weight determination and the infrared spectrum (Fig. 1) support the proposed formula, however.

Molecular Weights.—The new esters of anisic acid were titrated with 0.01 *N* sodium hydroxide in a 1:1 ethanol-water solvent; neutralization equivalents were determined using a Beckman pH meter, Model G. For the monoacidic esters, the neutralization equivalent equaled the molecular weight.

Infrared Spectra.—Infrared spectra were obtained using a Baird Infrared Recording Spectrophotometer, Model B, employing a sodium chloride plate. Nujol mulls were used for each compound. Nujol exhibits characteristic absorption peaks at 3.4–3.6, 6.8–6.9, 7.2, 8.6 and 13.8. Each of these peaks was visible in the newly prepared products and can be seen in the examples shown. (Figs. 1 and 2) The characteristic absorption peak for an ester linkage is found at 5.8–5.9; this band was visible in each spectrum. At wave lengths of 6.2, 6.6, and 7.9, each product exhibited the characteristic absorption peaks of the methoxy or the 1–4 substituted benzene groups. The amine hydrochloride peak is found at a wave length of 4.2; this band was exhibited by each of the new compounds.

Pharmacological Properties.—The authors are indebted to the Eli Lilly Company for the pharmacological testing of the new esters.

Preliminary tests indicate that all five new compounds are less than 5% as active as atropine in releasing methacholine spasm. Only one of the new esters, 2-diethylamino-1-methylethyl anisate hydrochloride, showed a trace of analgesia in rats in doses from 10 to 200 mg./Kg. Two of the compounds, 2-anilinoethyl anisate hydrochloride and 2-isopropylaminoethyl anisate hydrochloride, showed prolonged local anesthesia by the guinea pig skin test but produced some irritation.

SUMMARY

1. Five new derivatives of anisic acid were prepared by direct reaction of the amino alcohol with the acid chloride. Carbon-hydrogen and molecular weight determinations were performed upon each compound and compared with calculated, theoretical values. Comparison of infrared absorption bands of each compound with previously determined, characteristic bands verified the presence of the expected functional groups.

2. Pharmacological screening tests for analgesia, antispasmodic, and local anesthetic action were completed.

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Factors Affecting Tablet Disintegration*

By KING CHIU KWAN, FRED O. SWART, and ALBERT M. MATTOCKS

A 4 x 4 x 3 x 2 factorial experiment was run to determine the effects of disintegrating agents, lubricants, binders, and hardness on tablet disintegration. A single tablet filler, starch-lactose equal parts, was used. Tablets were found to disintegrate equally rapidly with or without disintegrating agent, thus allowing no comparison between disintegrating agents. Binders, lubricants, and hardness were found to differ in their effects on disintegration, and these factors are discussed. One two-factor interaction was found and shown to be due to a greater effect of hardness on tablets made with gelatin binder.

NUMEROUS EVALUATIONS of new agents as possible tablet disintegrators have been made (1–9, 11). The difficulties encountered in such studies were indicated by the experiments of several of these workers. Berry (2) found that tablet formula, size, and weight of tablets, size of granules, temperature, humidity, and type of hopper feed caused variations of disintegration rate. Burlinson and Pickering (6) found in their tests that pressure did not cause much variation

* Received July 9, 1955, from the Manufacturing Laboratories, College of Pharmacy, University of Michigan, Ann Arbor.

in disintegration time, while Higuchi, *et al.* (10), found an exponential relationship between compressional force and disintegration time. These workers also noted that different batches of the same formula disintegrated at different rates. Holstius and DeKay (9) studied tablet disintegration by a factorial experiment and included tablet composition, binders, and disintegrators as factors. Their experiment demonstrated that different materials are subject to different effects by various binders and disintegrators. This made it clear that results obtained with one tablet filler-binder combination cannot be expected to give results which can be extrapolated to another. It also indicated that any attempt to evaluate or compare disintegrating agents should include a study of response of the agents to changes in the various factors involved in tablet making.

It was our purpose to evaluate a new tablet disintegrator and to learn something of its behavior under varied conditions encountered in the normal process of tabletting.

To eliminate the variations due to tablet composition, it was decided to select a single tablet filler which might represent the widest scope possible and to use this as the basic ingredient for all tests. By using only one tablet filler it was possible to investigate in more detail the effect of binders, lubricants, hardness, and still get a direct comparison between two disintegrators.

The new material evaluated as a disintegrator is Solka Floc® BW-100.[1] This material is a highly purified alpha cellulose prepared from wood fiber. It is available in several sizes, this particular one, BW-100, having an approximate size of 45 microns. While previously used as a filter aid it has been used also as a tablet filler, lubricant, and disintegrator. Its fibrous nature and affinity for water would lead one to expect it to be effective as a tablet disintegrator, particularly in view of the explanation of starch disintegrating action based on capillarity (12). In order to measure the effect of Solka Floc it was decided to compare it to starch, the most commonly used disintegrating agent. Later it was decided to include a study where no disintegrating agent was present, thus obtaining not only a comparison between starch and Solka Floc but also a measure of their effectiveness.

EXPERIMENTAL

Tablets were prepared from one basic tablet filler, starch-lactose equal parts, this being selected as probably the most common and most versatile filler. Four binding solutions were used: 10% acacia, 10% starch paste, 10% gelatin, and 1% carboxymethyl cellulose-HV. The lubricants selected were stearic acid, calcium stearate, talc, and mineral oil, all used as 1% of the dried granules. The disintegrators were corn starch and Solka Floc, used as 10% of the dried granules.

The granulations were prepared from the same lots of ingredients, using the same quantities of binding solutions. Four large batches of granulation, one with each binder, were divided into portions to be mixed with the different combinations of lubricant and disintegrator. Four additional batches were prepared for the combinations containing no disintegrator.

Tablets were made at two levels of hardness, the hard as near a reading of 15, the soft as near 10 as possible on the Strong-Cobb hardness tester. These hardnesses were obtained simply by adjusting the pressure wheel on the single-punch tablet machine.

All possible combinations of these factors, binder, disintegrator, lubricant, and hardness were made, giving a 4 x 4 x 3 x 2 factorial experiment requiring ninety-six batches of tablets. Approximately two hundred tablets were made of each combination.

Disintegration rate was determined in seconds by the U. S. P. XV procedure, twelve tablets from each mixture being tested. Results are shown in Table I, and the analysis of variance of the results is shown in Table II.

DISCUSSION AND CONCLUSIONS

Preliminary calculations showed that the four factor interaction, BLDH, was significant when tested against residual obtained by using individual disintegration times (for single tablets). Thus, it was considered that the four factor interaction was a better estimate of experimental error. The analysis of variance was thus performed on the total disintegration time for the twelve tablets of each group, this making the four factor interaction the residual.

From the analysis of variance it is seen that no difference was shown between the disintegrating agents and that the tablets disintegrated as rapidly without a disintegrating agent. This result is surprising since the average disintegration times were not extremely low. If the rates had reached a minimum without disintegrating agent, say ten or fifteen seconds, it may be expected that addition of a disintegrating agent would not have a measurable effect. It is possible, however, that enough of the starch contained in the basic granulation had become powdered during the process to fall within the intergranular spaces of the tablets and thus give a maximum effect for this type of disintegrating agent, whereupon addition of more starch or another agent acting in the same manner would have no effect. This means that the experiment is not suitable for comparing the effectiveness of starch and Solka Floc as disintegrating agents. The results are of value, nevertheless, in demonstrating the effects of the other factors on disintegration time.

The effect of binders was significant, and a more detailed examination by means of orthogonal comparisons or *t* tests showed each binder to differ in its effect from the rest. Table III shows that starch paste gave fastest disintegration, with carboxymethylcellulose, acacia, and gelatin following in increasing order. The effect of binder on disintegration may be due to its rate of solution or dis-

[1] Available from the Brown Co., Berlin, New Hampshire.

TABLE I.—RESULTS OF DISINTEGRATION TESTS

Total Time in Seconds for Disintegration of 12 Tablets							
Combination	Disint. Time	Combination	Disint. Time	Combination	Disint. Time	Combination	Disint. Time
$B_1L_1D_1H_1$	4,737	$B_2L_1D_1H_1$	2,468	$B_3L_1D_1H_1$	5,759	$B_4L_1D_1H_1$	4,063
$B_1L_1D_2H_1$	7,208	$B_2L_1D_2H_1$	712	$B_3L_1D_2H_1$	7,855	$B_4L_1D_2H_1$	6,683
$B_1L_1D_3H_1$	4,435	$B_2L_1D_3H_1$	1,448	$B_3L_1D_3H_1$	8,071	$B_4L_1D_3H_1$	923
$B_1L_1D_1H_2$	3,090	$B_2L_1D_1H_2$	536	$B_3L_1D_1H_2$	2,979	$B_4L_1D_1H_2$	3,280
$B_1L_1D_2H_2$	3,154	$B_2L_1D_2H_2$	169	$B_3L_1D_2H_2$	2,099	$B_4L_1D_2H_2$	3,251
$B_1L_1D_3H_2$	4,065	$B_2L_1D_3H_2$	739	$B_3L_1D_3H_2$	4,242	$B_4L_1D_3H_2$	585
$B_1L_2D_1H_1$	4,310	$B_2L_2D_1H_1$	2,581	$B_3L_2D_1H_1$	6,712	$B_4L_2D_1H_1$	3,518
$B_1L_2D_2H_1$	7,329	$B_2L_2D_2H_1$	3,541	$B_3L_2D_2H_1$	10,311	$B_4L_2D_2H_1$	5,303
$B_1L_2D_3H_1$	5,421	$B_2L_2D_3H_1$	498	$B_3L_2D_3H_1$	11,247	$B_4L_2D_3H_1$	2,120
$B_1L_2D_1H_2$	3,672	$B_2L_2D_1H_2$	478	$B_3L_2D_1H_2$	2,434	$B_4L_2D_1H_2$	3,029
$B_1L_2D_2H_2$	3,493	$B_2L_2D_2H_2$	436	$B_3L_2D_2H_2$	2,155	$B_4L_2D_2H_2$	2,106
$B_1L_2D_3H_2$	4,757	$B_2L_2D_3H_2$	727	$B_3L_2D_3H_2$	2,918	$B_4L_2D_3H_2$	3,007
$B_1L_3D_1H_1$	3,723	$B_2L_3D_1H_1$	648	$B_3L_3D_1H_1$	2,966	$B_4L_3D_1H_1$	2,962
$B_1L_3D_2H_1$	3,510	$B_2L_3D_2H_1$	1,090	$B_3L_3D_2H_1$	4,195	$B_4L_3D_2H_1$	1,439
$B_1L_3D_3H_1$	3,766	$B_2L_3D_3H_1$	322	$B_3L_3D_3H_1$	6,607	$B_4L_3D_3H_1$	1,112
$B_1L_3D_1H_2$	2,258	$B_2L_3D_1H_2$	287	$B_3L_3D_1H_2$	1,241	$B_4L_3D_1H_2$	2,192
$B_1L_3D_2H_2$	1,603	$B_2L_3D_2H_2$	138	$B_3L_3D_2H_2$	3,359	$B_4L_3D_2H_2$	764
$B_1L_3D_3H_2$	2,864	$B_2L_3D_3H_2$	306	$B_3L_3D_3H_2$	1,832	$B_4L_3D_3H_2$	899
$B_1L_4D_1H_1$	3,849	$B_2L_4D_1H_1$	1,451	$B_3L_4D_1H_1$	7,329	$B_4L_4D_1H_1$	3,199
$B_1L_4D_2H_1$	4,763	$B_2L_4D_2H_1$	1,073	$B_3L_4D_2H_1$	3,559	$B_4L_4D_2H_1$	2,812
$B_1L_4D_3H_1$	4,827	$B_2L_4D_3H_1$	856	$B_3L_4D_3H_1$	10,253	$B_4L_4D_3H_1$	1,700
$B_1L_4D_1H_2$	3,321	$B_2L_4D_1H_2$	433	$B_3L_4D_1H_2$	2,532	$B_4L_4D_1H_2$	2,768
$B_1L_4D_2H_2$	2,668	$B_2L_4D_2H_2$	154	$B_3L_4D_2H_2$	2,723	$B_4L_4D_2H_2$	1,374
$B_1L_4D_3H_2$	4,423	$B_2L_4D_3H_2$	795	$B_3L_4D_3H_2$	6,060	$B_4L_4D_3H_2$	1,024

TABLE II.—ANALYSIS OF VARIANCE

Source	Sum Squares	D. F.	Mean Squares	F vs. R.	F vs. I[a]
Binders (B)	229,662,770.9	3	76,554,257.0		12.76[b]
Lubricants (L)	40,461,016.4	3	13,487,005.0		9.04[b]
Disintegrating Agents (D)	1,112,047.0	2	556,023.0		<1.0
Hardness (H)	87,869,833.6	1	87,869,833.6		25.13[b]
BL	7,955,796.6	9	883,977.4		1.60
BD	41,594,352.9	6	6,932,392.2		3.74
BH	42,848,596.7	3	14,282,865.6		7.70[b]
LD	13,149,402.4	6	2,191,567.1		1.47
LH	9,421,591.2	3	3,140,530.4		2.10
DH	5,828,227.7	2	2,914,113.9		1.74
BDH	11,142,939.4	6	1,854,156.6	3.3[b]	
LDH	8,954,458.4	6	1,492,409.7	2.7[b]	
BLH	10,447,511.3	9	1,160,834.6	2.1	
BLD	19,601,964.7	18	1,088,998.0	2.0	
Residual	9,962,635.2	18	553,479.7		
Total	539,977,925.4	95			

[a] F determined vs. combined mean square of significant interactions involved.
[b] Significant at 5% level.

persion in water. As can be seen by adding water to their dried films, rates of solution fall in the same order as do these tablet disintegration rates.

Lubricants were also found to have a significant effect on disintegration, with each being significantly different from the others. From Table III it is seen that increasing order of disintegration time for lubricants is talc, mineral oil, stearic acid, calcium stearate. The effect of lubricants is believed to be due to their making the tablet surfaces water-repellent, the lubricants themselves being insoluble.

Hardness of tablet was shown to affect disintegration rate considerably in these formulas. This would be expected from the formulas used in this experiment. In order for water to enter the tablet body there must be pores or capillaries, and the gran-

TABLE III.—SINGLE EFFECTS ON DISINTEGRATION

Disintegrators		Binders		Lubricants		Hardness	
Average Disintegration Time in Seconds							
Starch	246.9	Starch	76.0	Talc	173.9	Hard	339.0
Solka Floc	263.1	CMC	208.7	Mineral oil	256.8	Soft	177.1
None	267.8	Acacia	337.7	Stearic acid	286.6		
		Gelatin	414.7	Calcium stearate	319.8		

ules of these formulas are deformable under pressure, the granules yielding to fill in the pore spaces. Thus, the greater the pressure (hardness) the fewer pores in the tablets. This would not apply to all formulations, since harder granules, acting like hard crystals, might tend to fracture rather than deform under pressure and give perhaps less pore volume but a larger number of pores.

TABLE IV.—BH INTERACTION. INCREASE IN DISINTEGRATION TIME DUE TO INCREASED HARDNESS

Binder	Effect, sec.
Starch	79.8
CMC	80.2
Acacia	128.5
Gelatin	349.2

Only one two factor interaction, that of BH, was found to be significant. As seen in Table IV this result was due almost entirely to the effect of increased hardness on tablets containing gelatin. Although all the formulas showed increase in disintegration time with increased hardness, gelatin caused a much greater response than the other binders. One might speculate that this is due to the gelatin granules being considerably more plastic than the others, but this effect is not clear and requires more study.

The three factor interactions found to be significant, BDH and LDH, are highly complex and difficult to interpret. The authors, therefore, make no attempt to interpret them. Further experimentation, properly designed, may make these effects more understandable.

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The Pharmacology of Tetramine (Tetraethylenedisulfotetramine)*

By A. R. HASKELL† and ELBERT VOSS

Pharmacological studies have been made of Tetramine (tetraethylenedisulfotetramine), employing principally a saturated aqueous solution of the compound. Tetramine was found to be stable in aqueous solutions and the LD_{50} of this solution for the albino rat has been determined. The compound was shown to have no major activity on peripheral nerves, skeletal muscles, or the neuromuscular junction; investigations of autonomic activity were without significant positive results. Convulsive activity of Tetramine was shown to lie entirely within the brain stem. The compound greatly increased both rate and amplitude of respiration in the deeply anesthetized dog. Absorption studies indicate that Tetramine is rapidly absorbed from the mucosa of the pharynx and oral cavity; it appears to be metabolized at a rate approximately one-fourth the LD_{50} per day.

THE REACTION of sulfamide and formaldehyde to form a clear, stable resin was reported by Wood and Battye in 1933 (1). Audrieth, *et al.*, later reported the structure of this compound (2), which eventually came to be used as an impregnating, stiffening, and antimold agent for fibers used in pillows and upholstery.

An outbreak of severe poisoning which occurred in employees of certain German furniture-manufacturing industries was traced to "Crinex" wool which had been impregnated with this resin (3). Hagen's examination indicated that the causative agent was probably identical with the highly toxic compound Tetramine, the synthesis of which had been published by Hecht and Hen-

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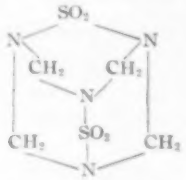
† Present address: Jensen-Salsbery Laboratories, Inc., Kansas City 41, Mo.

ecka (4) in 1940. The structural formula for Tetramine is reported to be as follows (3, 6):



Tetramine is a white powder which is very slightly soluble in water and acetone. It has been shown by Hagen to be approximately five times as toxic as strychnine for mice, the LD_{50} in the latter animal being 0.1–0.2 mg./Kg., and for albino rats, 0.22 mg./Kg. (3, 6). The LD_{50} for other common laboratory animals was reported to be of the same order of magnitude.

Earliest reports of the pharmacological activity of Tetramine concerned its convulsive properties, and the compound was soon proposed for use as a rodenticide (5). Other investigators soon discovered that Tetramine had pronounced repellent properties for rodents (7, 8, 9), and this discovery led to an investigation of its use as a rodent repellent in reforestation projects (8). This application would appear hazardous in view of the extreme toxicity of the compound, although it has been reported to be very effective in preventing rodent consumption of seedlings.

Platcow (6) undertook the initial study of Tetramine at the University of Florida. His work confirmed the LD_{50} as reported by Hagen for the albino rat. Platcow demonstrated the lack of significant histologic pathology from prolonged administration of sublethal doses and showed that Tetramine had only a low acceptability for rats when applied in the drinking water.

All previous investigations into the pharmacology and toxicology of Tetramine have demonstrated its extreme toxicity; detailed investigations of its pharmacology have not been reported.

Platcow used acetone as the solvent for intraperitoneal administrations of Tetramine. For general pharmacological testing, acetone is not a desirable solvent, its toxicity being greater than that of methyl alcohol (10, p. 629); therefore, water would appear to be more acceptable as a solvent for pharmacological studies.

EXPERIMENTAL

Animals.—For determination of the LD_{50} of the saturated aqueous solutions of Tetramine, we used albino rats of the Wistar strain, weighing between 100 and 150 Gm., obtained from J. C. Landis, Hagerstown, Maryland. Upon receipt in our laboratory they were maintained on a diet of Purina Laboratory Chow and water *ad libitum* for a minimum period of four days before use.

Dogs used in our studies were healthy mongrels which were observed for a minimum period of one week before use. Healthy adult alley cats were observed for gross defects for at least two days before use. For skin absorption studies, immature rabbits weighing between 500 and 1000 Gm. were employed. Healthy frogs (*Rana pipiens*) were employed in part of the work for determining the location of central nervous actions.

The Tetramine employed in this investigation consisted of a 5-Gm. sample labeled "Tetramine 100%," supplied by Mr. Donald A. Spencer, Wild Life Research Laboratories, Denver, Colorado.

EXPERIMENTAL METHODS

Tetramine Solutions.—A clear saturated aqueous solution was prepared and stored at room temperature in nonactinic containers from which portions were removed as needed immediately before use.

Determination of the LD_{50} of the Saturated Aqueous Solution.—Preliminary administrations of this solution to albino rats indicated that 2 ml./Kg., while convulsive in all cases, was never fatal, and that 7 ml./Kg. was fatal in all cases. Increments ranging between these amounts were administered intraperitoneally to five groups of eight rats each. Mortality tabulations were made at the end of 24 hours and plotted on semilogarithmic probability paper. The $(Chi)^2$, slope with 95% confidence limits, slope function of the line, and the LD_{50} and fLD_{50} were determined by statistical procedures outlined by Litchfield and Wilcoxon (11).

Determination of Stability of the Saturated Aqueous Solution.—The LD_{50} was used as an indication of stability of this solution. Previously standardized stock solutions were reexamined at intervals of one week, six weeks, and five months. Parallelism of the lines from each successive test was compared by calculation of the slope ratios by the method of Litchfield and Wilcoxon.

General Pharmacological Activity

Saturated aqueous solutions of Tetramine were used, unless otherwise specified, for studies of pharmacological activity.

Location of Convulsive Action, Frogs.—Four different frog gastrocnemius muscle preparations with sciatic nerve attached were employed. Stimuli were delivered to the nerve through platinum electrodes from a Grass stimulator set to deliver one stimulus of 5 msec. duration every three seconds. Variations of voltage showed a minimum response at approximately 0.12 volt and a maximum at approximately 0.6 volt. A response at 0.2 volt was taken as the test response. A normal response was recorded for each preparation bathed in frog saline, after which the latter was replaced by the saturated aqueous solution of Tetramine, and recordings continued; after 10 minutes the Tetramine solution was sprayed directly on the muscle. At the conclusion of each experiment a maximum response was recorded at 0.6 volt.

Sciatic Nerve Achilles Tendon Preparation, White Rat.—Eight rats were used in these studies.

After anesthetization with sodium pentobarbital, 50 mg./Kg., an achilles tendon of each rat was prepared in the usual manner for recording its contractions. In four of these animals the sciatic nerve was isolated with its central connections left intact. Stimuli were delivered to the nerve through electrodes attached to a Grass stimulator set to deliver 0.4 volt of 2 msec. duration at a rate of one stimulus every three seconds. A second group of 4 rats was utilized in which the preparation differed only by having the sciatic nerve cut before beginning the recording. Tetramine solution was administered intraperitoneally in a dose of one LD_{50} to each rat.

Action on the Central Nervous System.—*Frogs.*—Thirty-five frogs in seven groups of five frogs each were employed. Group one frogs were decerebrated; group two frogs were first decerebrated and optic lobes removed by careful dissection, and group three frogs were beheaded. Group four frogs were used as controls for the Tetramine injections. Five-tenths milligram of Tetramine dissolved in 0.5 ml. of acetone was injected into the ventral lymph sac of each frog of these groups, the test animals having recovered from shock before injections were given. After convulsions were well advanced in the controls, they were then treated by serial removals of brain segments, as were the test animals. Three orher groups of five frogs each were first prepared as for groups one, two, and three, respectively, and each frog was given 0.5 ml. of acetone intraperitoneally.

Decerebrate Cats.—Four decerebrate cats were prepared according to the method outlined by Burn (12, p. 35–38). After a suitable interval each animal was given 5 ml. of Tetramine solution intraperitoneally and observed for convulsive effects.

Spinal Cats and Dogs.—Three spinal cats and four spinal dogs were prepared by complete severance of the spinal cord between the second and third cervical vertebrae, the animals having been previously anesthetized with sodium pentobarbital, 35 mg./Kg., intraperitoneally. Blood pressure was recorded from the carotid artery, and respiration maintained through a tracheal cannula by means of a Palmer respiratory pump. To each spinal animal was administered a total dose of 10 ml. of the saturated aqueous solution of Tetramine by slow injection into the femoral vein.

Effect on Blood Pressure of Anesthetized Normal Dogs.—Several occasions were utilized for study of the effects of Tetramine in anesthetized dogs prepared for recording blood pressure and respiration. Tetramine solution was injected intravenously in initiai doses of 0.5 ml./Kg. Repetitions of these injections were frequently made until the animals demonstrated skeletal muscle spasms.

Effects on Kidney and Spleen Size.—These studies were made on anesthetized normal and spinal dogs, utilizing the methods of Jackson (13, p. 173), with sodium pentobarbital, 25 mg./Kg., intravenously, as the anesthetic. After a normal record, 0.5 ml./Kg. of Tetramine solution was administered through the femoral vein and the recording continued to observe any change in size of the organ. Spinal dogs were later given an intravenous dose of 2 ml./Kg. of the Tetramine solution to observe the effects of high doses on these organs.

Effect on Motility of the Intestine.—Anesthetized normal and spinal dogs were employed, having first been prepared as for studies on kidney and spleen size, except that a segment of ileum approximately 20 cm. above the pyloric valve was placed in an appropriate oncometer. Tetramine solution was injected into the femoral vein, and the records observed for any changes in size and motility of the gut segment. The normal anesthetized dogs received 0.5 ml./Kg. of the Tetramine solution; the spinal dogs received 1.5 ml./Kg.

Effect on Motility of the Bladder.—Four dogs, anesthetized as for studies on kidney and spleen, were prepared according to the method of Jackson (13, p. 217) for determining effects of Tetramine on bladder activity. After suitable control records, 0.5 ml./Kg. of Tetramine solution was injected into the femoral vein. When contraction of skeletal muscles became sufficient to alter the records, the animals were given 3 mg. *d*-tubocurarine intravenously and the records further observed for bladder contractions.

Effect on Respiration in Pentobarbital Anesthetized Dogs and Pentobarbital Poisoned Dogs.—Four animals were prepared for recording respiration and blood pressure in the usual manner. They were first given 0.5 ml./Kg. of Tetramine solution. After suitable control records the anesthetic was infused intravenously until respiration stopped and the blood pressure had fallen to approximately spinal levels. They were then given 0.75 ml./Kg. of Tetramine solution and resulting changes in blood pressure and respiration were recorded.

Absorption from the Intact Skin.—Hair was clipped from the thorax, abdomen, and insides of the thighs of six rabbits. A 60-mm. watch glass was taped to the depilated abdomen of each animal; they were individually caged and six daily applications of Tetramine solution were placed inside the watch glasses. After four days these animals were again prepared as follows: In three, a clean gauze of suitable size was placed between the watch glass and the skin and kept saturated with Tetramine solution for four hours. The three remaining animals were prepared in the same manner, with the exception that before gauze was applied the skin was carefully washed with liquid soap and water to remove natural oils.

Absorption from the Mucous Membrane.—Four dogs were anesthetized with sodium pentobarbital, 25 mg./Kg., and strapped on the surgery table with the head considerably elevated from the normal position and clamped only by the upper jaw. Through a longitudinal incision in the neck, the esophagus was doubly ligated at the level of the larynx and severed between the ligatures. The trachea was cannulated in the usual manner for controlled respiration and clamped above the cannula with a large hemostat. The upper esophagus, upper trachea, pharynx, and oral cavity were then filled with Tetramine solution and observed for increased respiration, muscle twitchings, convulsions or other signs of Tetramine effect. Control tests were made using water in place of Tetramine to demonstrate the absence of response of the cough and swallowing reflexes at this level of anesthesia.

Rate of Detoxication.—Two groups of eight rats each were employed in determining an approximate rate of detoxication of Tetramine. One group received one-half LD_{50}, and the other group one-fourth LD_{50} per day. All injections were administered intraperitoneally for a period of two weeks in surviving animals. At the end of fourteen days the dose for survivors of the second group was increased to one-half LD_{50} per day and injections continued through a total of 21 days. Death of the test animal during these periods was interpreted as indication that the drug was being given at a rate which exceeded that of its detoxication.

SUMMARY AND DISCUSSION OF RESULTS

The LD_{50} and Stability of Saturated Aqueous Solutions.—The LD_{50} of freshly prepared saturated aqueous solution was shown to be 4.85 ml. (4.25 to 5.5 ml./Kg.) in the albino rat. This figure indicates that Tetramine is sufficiently soluble in water to permit use of its saturated aqueous solutions for pharmacological studies. Further tests on stored solutions indicate stability of such solutions for a minimum period of five months.

General Pharmacological Activity.—Tetramine exhibited no major activity on peripheral nerve, skeletal muscle, or the neuromuscular junction, and no perceptible autonomic activity on spleen, kidney, intestine, or bladder.

The convulsive activity of Tetramine was shown to lie entirely in the brain stem, since removal of the cerebrum failed to stop the convulsions, while severance of the central nervous system below the medulla stopped spasms below the level of the lesion. An exacerbation of convulsions was observed in poisoned animals after removal of the cerebrum.

Tetramine greatly increased both rate and amplitude of respiration in dogs under surgical anesthesia. In animals intoxicated with pentobarbital to the point of cessation of respiration and precipitous drop in blood pressure to the spinal level, doses of 0.5 ml./Kg. brought about return of the respiration and increase in blood pressure.

Our studies on absorption indicated that Tetramine was absorbed rapidly from the mucous membrane of the upper respiratory and oral cavities. Use of the end points described for this study requires that toxic doses of drug be absorbed before termination of the experiment. Toxic doses were absorbed and the end point reached on an average of thirty minutes. Our studies on rabbits, however, indicated no intoxication from Tetramine solutions applied to the intact skin, even in animals whose skins had been previously washed with soap and water. The death of one animal in which there were several abrasions over the area to which Tetramine was applied would indicate the necessity of wearing rubber gloves when working with Tetramine solutions if lesions exist in the integument of the hands.

The number of animals dying from administrations of one-half LD_{50} of the Tetramine solution per day indicated that this dose is in excess of the daily rate at which it is destroyed; seven animals out of a group of eight were dead at the end of the fourth day and none survived the completion of this experiment. The number of animals dying from the administration of one-fourth LD_{50} per day indicated that this dose represents administration at a rate somewhat in excess of that at which it is destroyed or inactivated, since two animals in this series were dead at the end of the fourteenth day of the experiment.

No significant tolerance to the lethal effect of one-half LD_{50} of Tetramine per day was demonstrable in surviving animals previously treated for fourteen days with one-fourth LD_{50} per day.

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The Effect of Various Drugs upon the Antibacterial Activity of Erythromycin in Ointments*

By RANDALL B. TINKER† and WILLIAM J. HUSA‡

The effect of various medicinal agents and various ointment base constituents upon the antibacterial activity of erythromycin in petrolatum-type bases has been determined. Clinical trial of mixtures of erythromycin and other medicinal agents in ointments appears to be warranted on the basis of *in vitro* tests.

THE PHYSICIAN often encounters infections in which he would like to prescribe a particular antibiotic material in conjunction with some other medicinal agent. He is, however, reluctant to do so because of the lack of definite information regarding the efficiency and stability of the particular combination he wishes to prescribe.

This paper supplies some basic information for the dermatologist and the general practitioner with respect to the efficiency and stability of erythromycin when mixed with various ointments of the petrolatum-type base.

EXPERIMENTAL

Materials.—Powdered erythromycin[1] having a potency of 931 mcg./mg. was used. Chemicals and ointment constituents were of official grade. Dehydrated Penassay seed and base agar were used as media. The inoculum was prepared from a 24-hour culture of *Micrococcus pyogenes* var. *aureus* (ATCC 10,537), standardized, and 1 ml. of the suspension was added to each 125 ml. of seed agar at 45°. Twenty milliliters of base agar was placed in each flat-bottomed Petri dish and allowed to harden, after which 4 ml. of inoculum was poured evenly over the base agar surfaces (1, 2).

Evaluating the Ointments.—Sterile filter paper disks were used to place the ointment in contact with the seeded plate surface. The disks were held with sterile forceps and a small amount of ointment was placed on the smooth surface of the disk. Rotating the disks 10 times clockwise and 10 times counterclockwise on a piece of sterile glassine paper was found to apply approximately 20 mg. of ointment per disk. Excess ointment was removed from the edge of the disk prior to placement upon the test surface. Pressure was applied across the diameter of the disk in such a manner that complete and intimate contact with the test surface was attained.

After incubation for a period of 20–22 hours at 35–37°, the zones of inhibition were measured by means of a divider. One point of the divider was placed at the edge of the zone of inhibition and the divider was extended across the diameter of the disk to the edge of the zone of inhibition on the opposite side. Readings were then made directly from a graduated metric scale. In order to determine the

TABLE I.—THE EFFECT OF VARIOUS OFFICIAL OINTMENT BASE CONSTITUENTS ON THE ANTIBACTERIAL ACTIVITY OF ERYTHROMYCIN IN WHITE PETROLATUM

Percentage of Constituent	Mean Zone Reading in mm. After 0 week	1 week	4 weeks	12 weeks	24 weeks
Standard plate controls	3.0	3.7	3.3	3.1	2.9
	0.0	0.0	0.0	0.0	0.0
White Wax					
1	2.9	3.6	3.1	3.1	2.6
2	2.8	3.6	2.6	2.3	1.4
5	1.8	2.6	2.1	1.4	1.1
10	1.6	1.8	1.1	1.0	tr
Yellow Wax					
1	2.9	3.7	3.1	3.1	2.6
2	2.9	3.7	2.8	2.2	1.3
5	1.6	3.7	3.6	1.5	1.1
10	1.4	2.1	1.9	tr	tr
Wool Fat					
1	3.0	3.7	3.2	3.1	2.9
2	2.9	3.7	3.2	3.0	2.8
5	3.0	3.6	3.4	3.1	2.9
10	3.0	3.7	3.2	3.1	2.9
20	2.9	3.6	3.3	3.1	2.8
50	3.0	3.7	3.2	2.9	2.9
Yellow Petrolatum					
1	2.9	3.7	3.3	3.1	2.9
2	3.0	3.9	3.2	3.1	2.9
5	3.0	3.7	3.3	3.1	2.9
10	3.0	3.7	3.4	3.0	2.9
25	3.0	3.8	3.2	3.1	3.0
50	2.9	3.9	3.3	3.1	3.0
90	2.9	3.8	3.4	3.1	2.9
100	3.0	3.9	3.4	3.0	2.9
Glycerin					
1	2.8	3.7	3.3	3.1	2.9
2	2.9	3.6	3.3	3.1	2.9
5	3.1	3.7	3.3	3.1	2.9
10	3.1	3.6	3.3	3.0	2.9
Liquid Petrolatum					
1	3.0	3.7	3.3	3.1	2.9
2	3.1	3.6	3.2	3.2	2.9
5	3.1	3.7	3.3	3.1	2.9
10	3.1	3.7	3.3	3.2	2.9
Water					
1	3.1	3.6	3.4	3.3	2.9
2	3.1	3.5	3.3	3.1	2.9
4	3.1	3.8	3.4	3.1	2.9

* Received February 8, 1956, from the College of Pharmacy, University of Florida, Gainesville.
This paper is based in part upon a dissertation presented to the Graduate Council of the University of Florida by Randall B. Tinker, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
† Fellow of the American Foundation for Pharmaceutical Education. Interim Assistant Research Professor, College of Pharmacy, University of Florida.
‡ Head, Department of Pharmacy, University of Florida, Gainesville.
[1] The erythromycin used in this study was generously supplied by Eli Lilly and Company, Indianapolis, Ind.

width of the zone of inhibition, the diameter of the disk (13 mm.) was subtracted and the difference was divided by two. This gave the distance from the edge of the disk to the periphery of the zone of inhibition. It was felt that this method of calculation gave a more nearly accurate measurement than directly measuring from the edge of the disk to the edge of the zone of inhibition.

The mean zone of inhibition produced by each ointment was compared with the mean inhibition zone obtained from a standard plate made concurrently with each test run.

Selection of Erythromycin Concentration.—An ointment containing 10 mg. of erythromycin per Gm. of ointment was prepared in white petrolatum and from this, ointments containing 0.5, 1, 2, and 5 mg./Gm. were prepared. These ointments were tested against the test organism, the zones of inhibition were recorded and averaged and the results were plotted on coordinate paper. The resulting curve was examined to determine the concentration at which a minimal change in erythromycin concentration produced a maximal change in the size of the zone of inhibition. This concentration was determined to be between 0.5 and 1 mg./Gm., and since deterioration of the ointments was to be measured, the latter concentration was selected for use in the preparation of the various ointments for test.

Erythromycin Standard Plate.—Inasmuch as preliminary experiments had shown that inconsistencies in readings were obtained when a standard curve was used for reference, one standard plate was used to determine the reference mean for each test run.

Effect of Ointment Base Constituents.—Several series of ointments were prepared according to accepted pharmaceutical techniques, each series containing varying concentrations of each of the following materials found in the official ointment bases: white wax, yellow wax, wool fat, yellow petrolatum, liquid petrolatum, glycerin, and water. These ointments were tested as previously described and the results shown in Table I were obtained.

The results in Table I show that a concentration of white or yellow wax of 5 per cent or greater decreased the zone of inhibition significantly immediately upon being incorporated into erythromycin-containing ointments. At the end of one month a similar effect was shown with only 2 per cent of either wax, while at the end of six months, a concentration of 1 per cent of either wax produced a significant decrease in zone size. No effect on the antibacterial activity of the erythromycin was produced by any of the other base constituents in any of the concentrations tested. These conclusions were substantiated by statistical evaluation using

TABLE II.—THE EFFECT OF VARIOUS MEDICINAL AGENTS UPON THE ANTIBACTERIAL ACTIVITY OF ERYTHROMYCIN IN WHITE PETROLATUM

Ointment	Mean Zone Reading in mm. After 0 week	1 week	4 weeks	12 weeks	% Antibacterial Activity[a] After 0 week	1 week	4 weeks	12 weeks
Standard plate	3.1	3.3	3.0	3.1	100.0	100.0	100.0	100.0
Ammoniated mercury control	2.4	2.4	2.1	2.2	77.4	72.7	70.0	71.0
Ammoniated mercury with erythromycin	4.0	4.0	3.4	3.1	129.0	121.1	113.3	100.0
Benzoic and salicylic acids, control	2.4	2.6	2.3	2.3	77.5	78.8	76.7	74.1
Benzoic and salicylic acids, with erythromycin	4.6	4.2	2.9	2.9	148.4	127.3	96.7	93.5
Boric acid control	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Boric acid with erythromycin	2.9	2.5	1.9	1.6	93.5	75.8	63.3	51.6
Calamine control	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Calamine with erythromycin	3.5	3.5	2.9	2.9	112.9	106.1	96.7	93.5
Ethyl aminobenzoate control	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Ethyl aminobenzoate with erythromycin	2.3	2.3	1.9	1.9	74.2	69.7	63.3	61.3
Mercury bichloride control	t	t	t	t	t	t	t	t
Mercury bichloride with erythromycin	3.1	3.3	2.9	2.9	100.0	100.0	96.7	93.5
Mild mercurous chloride control	2.1	2.1	1.9	1.9	67.6	63.6	63.3	61.3
Mild mercurous chloride with erythromycin	3.9	4.1	3.5	3.3	125.8	124.2	116.7	106.5
Phenol control	1.6	1.7	1.5	1.2	51.6	51.5	50.0	38.7
Phenol with erythromycin	3.6	2.1	1.3	1.1	116.1	63.6	43.3	35.5
Red mercuric oxide control	t	t	t	t	t	t	t	t
Red mercuric oxide with erythromycin	2.8	2.9	2.5	2.3	90.3	87.9	83.3	74.2
Yellow mercuric oxide control	1.4	1.5	1.3	1.3	45.2	45.5	43.4	41.9
Yellow mercuric oxide with erythromycin	3.1	3.2	2.6	2.4	100.0	96.9	86.7	77.4
Zinc oxide control	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Zinc oxide with erythromycin	3.6	3.3	2.9	2.9	116.1	100.0	96.7	93.5

[a] Zone of inhibition produced by 1 mg./Gm. erythromycin in white petrolatum taken as 100.0%.
[t] Trace of inhibition; zone too small to measure.

the Student-Fisher "T" test for significance at the 95 per cent probability level. The mean zone of inhibition obtained from the standard plate was taken as the population mean.

Effect of Various Medicinal Agents.—The medicinal agents of several of the official ointments were incorporated into white petrolatum by levigation and geometric dilution. The amount of medicinal substance used in each ointment corresponded to that concentration found in the official ointment. These ointments were tested as previously described and the results obtained are shown in Table II.

The data in Table II show that when erythromycin in white petrolatum base was mixed with ammoniated mercury, benzoic and salicylic acids, calamine, mild mercurous chloride, phenol, and zinc oxide, the antibacterial activity of the mixture was greater than that of either the antibiotic or other medicinal agent alone.

The activity of the ointments containing erythromycin with boric acid, ethyl aminobenzoate, and red mercuric oxide was greater than that of the various medicinal agents without the erythromycin although it was not as great as the antibiotic control ointment. Mercury bichloride and yellow mercuric oxide in the erythromycin ointments produced the same zone of inhibition as did the antibiotic control.

The data in Table II further indicate that the antibacterial activity of all ointments containing erythromycin in the presence of other drugs was significantly reduced after an aging period of 12 weeks. At the end of 1 week, however, the ointments containing both erythromycin and ammoniated mercury, benzoic and salicylic acids, calamine, mild mercurous chloride, mercury bichloride, and zinc oxide, in white petrolatum showed an antibacterial activity equal to or greater than that of the antibiotic control ointment. Over a period of 4 weeks the ointment containing ammoniated mercury and erythromycin showed a greater activity than the antibiotic control ointment while mild mercurous chloride and erythromycin ointment exhibited an activity greater than the erythromycin control ointment over a period of 12 weeks. These conclusions were supported by statistical evaluation.

TABLE III.—THE EFFECT OF VARIOUS OFFICIAL OINTMENTS ON THE ANTIBACTERIAL ACTIVITY OF ERYTHROMYCIN

Ointment	Mean Zone Reading in mm. After 0	1	4	12	24[b]	Percentage Antibacterial Activity[a] After 0	1	4	12	24[b]
Standard plate	3.0	3.7	3.3	3.1	2.9	100.0	100.0	100.0	100.0	100.0
Ammoniated mercury control	2.5	3.0	2.7	2.4	2.2	83.3	81.1	81.8	77.4	75.9
Ammoniated mercury with erythromycin	3.9	4.1	3.6	3.3	3.0	130.0	110.8	109.1	106.5	103.4
Benzoic and salicylic acids, control	2.4	2.9	2.6	2.3	2.1	80.0	78.4	78.8	74.2	72.4
Benzoic and salicylic acids, with erythromycin	4.6	4.3	3.2	3.0	2.8	153.3	116.2	96.9	96.8	96.6
Boric acid control	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Boric acid with erythromycin	2.9	2.9	2.1	1.9	1.7	96.7	78.4	63.6	61.3	58.6
Ethyl aminobenzoate control	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Ethyl aminobenzoate with erythromycin	2.2	2.6	2.1	1.9	1.7	73.3	70.3	63.6	61.3	58.6
Mercury bichloride control	...[c]	...[c]	...[c]	...[c]	...[c]	...[c]	...[c]	...[c]	...[c]	...[c]
Mercury bichloride with erythromycin	3.1	3.7	3.3	3.0	2.8	103.3	100.0	100.0	96.8	96.6
Phenol control	1.8	2.1	1.5	1.3	1.2	60.0	56.8	45.5	41.9	41.4
Phenol with erythromycin	3.6	2.4	1.4	1.3	1.2	120.0	64.9	42.5	41.9	41.4
Yellow mercuric oxide control	1.8	2.1	1.6	1.4	1.3	60.0	56.8	48.5	45.2	44.8
Yellow mercuric oxide with erythromycin	3.1	3.6	2.9	2.6	2.4	103.3	97.3	87.9	83.9	82.8
Zinc oxide control	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Zinc oxide with erythromycin	3.5	3.8	3.3	3.0	2.8	116.7	102.7	100.0	96.8	96.6
Standard plate[c]	3.0	3.3	3.6	3.1	3.1	100.0	100.0	100.0	100.0	100.0
Calamine control	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Calamine with erythromycin	3.6	3.9	3.4	3.1	2.9	120.0	105.4	103.0	100.0	93.5
Mild mercurous chloride control	2.1	2.4	2.2	1.9	1.9	70.0	64.9	63.6	61.3	61.3
Mild mercurous chloride with erythromycin	3.9	4.6	3.9	3.6	3.3	130.0	124.3	118.2	116.1	113.8
Red mercuric oxide control	...[c]	...[c]	...[c]	...[c]	...[c]	...[c]	...[c]	...[c]	...[c]	...[c]
Red mercuric oxide with erythromycin	2.7	3.2	2.8	2.6	2.4	90.0	86.5	84.9	83.9	82.8

[a] Zone of inhibition produced by 1 mg./Gm. erythromycin in white petrolatum taken as 100.0%.
[b] Aging time in weeks.
[c] Limitation of incubator space required this data to be accumulated in two runs.

Effect of Various Official Ointments.—The eleven ointments shown in Table III were prepared according to the directions in the official compendia and to one-half of each ointment, 1 mg./Gm. of erythromycin was added, the other half of the ointment serving as a control. These ointments were tested as previously described.

The data in Table III show that when powdered erythromycin was incorporated with the official ammoniated mercury, benzoic and salicylic acids, calamine, mercury bichloride, mild mercurous chlo-

ride, yellow mercuric oxide and zinc oxide ointments, the antibacterial activity of the mixture was greater than that of either the official ointment alone or the antibiotic control ointment.

The antibacterial activity of erythromycin when mixed with boric acid, ethyl aminobenzoate, and red mercuric oxide ointments was greater than that of the official ointment without the erythromycin but not as great as that of the antibiotic control ointment.

These data further indicate that an aging period of 24 weeks significantly reduced the antibacterial activity of all the erythromycin-containing ointments except the antibiotic control ointment. The ointments containing ammoniated mercury, benzoic and salicylic acids, calamine, mercury bichloride, mild mercurous chloride, and zinc oxide in the official vehicles to which erythromycin was added showed an antibacterial activity equal to or greater than that of the antibiotic control ointment at the end of 1 week. Calamine, mercury bichloride and zinc oxide ointments with added erythromycin showed an activity equal to that of the control ointment after 4 weeks aging while ammoniated mercury and mild mercurous chloride ointments with added erythromycin maintained a portion of their increased activity over a period of 24 weeks. These conclusions were supported by statistical evaluation of the data.

DISCUSSION

A dermatologist or general practitioner seldom allows the elapse of more than 1 week between patient observations when a condition which requires the application of medicaments in ointment form is being treated. Therefore the unstable medications may be compounded only in sufficient quantity for application between patient observations.

Tables II and III of this paper show that the antibacterial activity of the erythromycin is significantly enhanced by incorporation of the antibiotic material with ammoniated mercury, benzoic and salicylic acids, calamine, mild mercurous chloride, phenol and zinc oxide in white petrolatum as well as in the official ointment base for each drug. Most of the enhanced activity of erythromycin is maintained for a period of one week except with phenol and zinc oxide; phenol produces an antagonistic effect while zinc oxide shows no particular advantage over the antibiotic ointment alone.

Mercury bichloride and yellow mercuric oxide have no initial effect upon the activity of erythromycin while boric acid, ethyl aminobenzoate, and red mercuric oxide decrease the activity significantly.

There is a significant decrease in the antibacterial activity of all ointments containing erythromycin except the control ointment. A portion of the enhanced activity of erythromycin is maintained over a 12-week period when it is mixed with ammoniated mercury and with mild mercurous chloride.

Clinical trial of mixtures of erythromycin with other medicinal agents in ointment form appears to be warranted upon the basis of these *in vitro* tests.

SUMMARY

1. The effect of various medicinal agents on the antibacterial activity of erythromycin in petrolatum-type base ointments has been determined. The following drugs enhanced the activity of erythromycin up to and including a period of 1 week: ammoniated mercury, benzoic and salicylic acids, calamine, and mild mercurous chloride.

2. The activity of erythromycin in petrolatum-type base ointments was not affected over a period of 1 week by the following: mercury bichloride, yellow mercuric oxide, and zinc oxide.

3. The activity of erythromycin was destroyed in 1 week by the following medicinal agents: boric acid, ethyl aminobenzoate, phenol, and red mercuric oxide.

4. The following ointment-base constituents had no effect on the antibacterial activity of erythromycin over a period of 6 months when mixed with white petrolatum in the concentrations used in the official ointment bases: white petrolatum, yellow petrolatum, liquid petrolatum, wool fat, glycerin, and water.

5. White and yellow wax in concentrations of 5 per cent or more rapidly destroyed the antibacterial activity of erythromycin in white petrolatum.

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Use of Ion Exchange in Recovery and Partial Purification of Morphine from Aqueous Extracts of Poppy Capsules*

By T. A. McGUIRE, C. H. VAN ETTEN, F. R. EARLE, and F. R. SENTI

A cation-exchange resin has been used successfully to recover morphine from aqueous extracts of poppy capsules. With 65 ml. of extract containing 65 mg. of morphine applied per gram of resin, approximately 94 per cent of the morphine was recovered when the resin was in the sodium form and 89 per cent when in the ammonium form. The resin showed no loss of capacity after 21 cycles. The crude solid product from the column eluate contained about 54 per cent morphine. By passing the eluate from the ammonium resin through an anion exchange resin, a solid product containing 85 per cent morphine was obtained.

MEHLTRETTER AND WEAKLEY (1) have described a procedure for recovering morphine from isobutanol extracts of dried poppy capsules by means of cation exchange resins. The alkaloid was eluted from the resin with aqueous alkali and crystallized by conventional methods.

During analysis of process samples, we observed that resins preferentially removed morphine from aqueous solutions containing relatively large amounts of inorganic cations. This paper presents results of a study of the recovery of morphine from aqueous extracts paralleling the essential steps of the previous work on alcoholic extracts.

MATERIALS AND METHODS

Poppy "straw," harvested by combine, was crushed between rolls and screened to eliminate stems. The capsular material was ground to about 20 mesh.

Distilled water was used for all operations.

In the laboratory, a repeated "soak-squeeze" procedure with hot water on a Buchner funnel resulted in extraction of 95 per cent of the morphine from ground capsular material in 15 minutes. In preparing the extract used for the recovery study, a commercial type extractor was used without any attempt to discover optimum conditions. The extract contained 1 mg. morphine per ml. and was stored at 1° with toluene as preservative. The pH was 5.1. Titration of acid in the extract before and after passage through an ion-exchange resin in the hydrogen form showed the extract to be 0.09 *N* with respect to acid and 0.26 *N* with respect to total cations. Unidentified solids separated during storage and were centrifuged out with little loss of morphine.

Duolite® C-10 cation-exchange resin was used for the recovery of morphine from the extract. Further purification was effected with the anion resin, Dowex® 1 X 1.

The cation exchange column consisted of a glass tube, 1.86 cm. i. d., with a fritted glass disk sealed in near the bottom. A Y-tube at the outlet permitted convenient collection of fractions and reclassification of the column by backwashing. The resin bed contained 27.6 Gm. (dry basis, sodium form) of resin and, in water, was 50 cm. deep. Under the influence of the extract, the bed contracted to 45 cm. but returned to its original height after elution of the morphine and reclassification of the bed. Flow rate was between 11 and 16 ml./min.

The anion column, 1.3 cm. i. d., was similarly constructed but contained only 10.3 Gm. (dry basis, chloride form) of resin. In the hydroxide form, to which the resin was converted immediately before use, the bed was 16 cm. deep. The bed contracted to 13 cm. depth in use but returned to its original depth on regeneration and reclassification.

For determination of morphine, the analytical method used was essentially that of Adamson and Handisyde (2). Before developing color with nitrous acid, the sample was exchanged on and eluted from a cation exchange resin to eliminate nonmorphine reactants. Comparison with the method of Van Etten, *et al.* (3), (Table I) shows that our purification was inadequate on most samples. The results, however, are consistent within themselves and are adequate for this study.

TABLE I.—COMPARISON OF METHODS OF ANALYSIS

Sample	—Weight of Morphine, mg.— Method Used	Van Etten's Method (3)	Ratio, %
Cation Column			
Influent	1760	1330	76
Effluent	40	25	63
Eluate	1650	1210	73
Anion Column			
Effluent	145	80	73
Eluate	1300	1110	85
Crystalization Procedure			
Mother liquor	220	180	82
Solid product	870	870	100

* Received September 18, 1956, from the new name: Northern Utilization Research and Development Division, Agricultural Research Service, United States Department of Agriculture, Peoria, Ill.

The authors wish to thank O. L. Brekke for making the pilot-plant extraction.

Mention in this article of firm names of commercial products does not constitute an endoresment of such firms or products by the U. S. Department of Agriculture.

TABLE II.—MORPHINE RECOVERY FROM DUOLITE C-10 (SODIUM FORM)

Run No.	Morphine in Influent, mg.	Adsorption, %	Elution, %[a]
1	2530	92	98
2	1740	97	104
3	2040	95	100
4	1850	96	103
5	1850	96	102
6	1860	95	103
7	1820	95	102
8	1820	89	102
9	1810	95	101
19	1770	93	103

[a] Based on adsorbed morphine.

EXPERIMENTAL AND RESULTS

Data on recovery of morphine from the water extract with Duolite C-10 in the sodium form are shown in Table II. The volume of influent varied during the first three runs but was maintained at 1,800 ml. for all succeeding runs. With this volume, about 95% of the morphine was retained by the resin, an arbitrary criterion of acceptable operation. During the first 6 cycles, the concentration and quantity of sodium hydroxide used for elution were varied.

In a typical run after No. 6, 1,800 ml. of extract was passed through the column at a rate of 11–16 ml./min. Spent extract was flushed from the column with 200 ml. of water. Morphine was eluted with 75 ml. of 1 *N* NaOH followed by 500 ml. of water. Reclassification by backwashing with water prepared the column for the next cycle.

The distribution of soluble solids was determined for run 9. Table III shows that 95% of the soluble material passed directly through the column and that 4% was recovered in the eluate. Even though 1% would seem to remain in the column, no noticeable decrease in the capacity of the column for morphine was experienced in the series of 21 cycles.

Results of operation of the resin in the ammonium form are shown in Table IV. The procedure was the same as that used with the sodium-form column except that 500 ml. of 1 *N* aqueous ammonia was used in elution. The behavior of the column was satisfactory although the capacity for morphine was reduced by about 5%.

Application of the eluate from the ammonium-form column directly to an anion-exchange resin, Dowex 1 X 1, in the hydroxide form resulted in removal of morphine and other amphoteric substances from the solution. Codeine passed through the column. Morphine with relatively few contaminants was eluted from the column with 150 ml. of 1 *N* HCl followed by 350 ml. of water. Results of four runs, shown in Table V, were somewhat variable but show promise of successful application.

TABLE III.—DISTRIBUTION OF TOTAL SOLUBLES OBTAINED FROM RUN 9

Material	Weight, Gm.	Per Cent
Influent	79.92	100.0
Effluent	74.40	93.1
H_2O wash before elution	1.60	2.0
Eluate[a]	2.84	3.6
H_2O wash after elution	0.20	0.3
Total Recovered	79.04	99.0

[a] Corrected for weight of Na_2SO_4 introduced during elution and neutralization.

TABLE IV.—MORPHINE RECOVERY FROM DUOLITE C-10 (AMMONIUM FORM)

Run No.	Morphine in Influent, mg.	Adsorption, %	Elution, %[a]
10	1810	96	93
11	1770	93	97
12	1750	88	90
13	1790	90	85
14	1780	90	92
15	1790	86	99
16	1780	84	100
17	1780	85	103
18	1780	83	106
20	1760	94	100
21	1770	90	102

[a] Based on adsorbed morphine.

TABLE V.—MORPHINE RECOVERY BY DOWEX 1 FROM AMMONIUM ELUATE FROM DUOLITE C-10

Run No.	Morphine in Influent, mg.	Adsorption, %	Elution, %[a]
11	1400	90	90
12	1250	96	98
13	1240	92	107
20	1570	91	91

[a] Based on adsorbed morphine.

Eluates from the Duolite C-10 column for runs 19 and 21 and from the Dowex 1 column for run 20 were neutralized to pH 7 and concentrated under vacuum to 60 ml. The solutions were then adjusted to pH 9.3 at 50° with ammonia and allowed to stand at 5° for 48 hours. The products from the sodium form and the ammonium form of the resin were essentially the same and contained 54% morphine. The effectiveness of the purification by the anion resin is evidenced by a morphine content of 85% in the product from the anion eluate.

CONCLUSION

Morphine can be extracted rapidly from poppy capsules with hot water It can be recovered from the extract by Duolite C-10 in either the sodium or ammonium form. The resin shows no appreciable loss of capacity during 21 cycles. Eluates from the resin are suitable for further processing by conventional methods and in the case of the ammonium-form resin by application of anion exchange.

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A Study of the Release of Selected Antibiotics from Cation-Saturated Bentonites*

By AUGUST G. DANTI† and EARL P. GUTH‡

The release of neomycin sulfate, oxytetracycline hydrochloride, and tetracycline hydrochloride from various prepared cation-saturated bentonites was determined by an agar cup plate method against the test organism *Micrococcus pyogenes* var. *aureus* (ATCC6538).

BENTONITE has been under investigation as a pharmaceutical agent for some time, including its capacity for releasing medicaments administered in conjunction with it. Bentonite is a member of the montmorillonite group of clays, its name derived from the mineral montmorillonite, having the composition $(OH)_4Al_4Si_8O_{20}.xH_2O$ and possessing a high base-exchange capacity.

Darlington and Guth (1) showed that the inclusion of bentonite as a major component of several emulsion and adsorption type bases and ointments increased their efficacy.

Sulfathiazole, ammoniated mecury, and phenol were demonstrated by Barr and Guth (2) to produce greater antibacterial activity when incorporated into a hydrogen bentonite than in several other cation-saturated bentonites.

Brockemeyer and Guth (3) showed that hydrogen and Volclay®[1] bentonite bases were more efficient in releasing cyclomethycaine[2] than several U. S. P. bases.

This investigation was undertaken to determine the effect of various cation-saturated bentonites on the release of neomycin sulfate,[3] oxytetracycline hydrochloride,[4] and tetracycline hydrochloride,[5] employing an *in vitro* agar cup plate method. The following abbreviations for the antibiotics will be used in the tables: NS, OHCl, THCl.)

EXPERIMENTAL

Preparation and Assay of the Various Cation-Saturated Bentonites.—Na, K, Mg, Ca, and H bentonites were prepared from Volclay bentonite[6] according to the method described by Barr and Guth (4). When referring to the various bentonites as Na, Ca bentonite, etc., it indicates the predominance of that cation in the bentonite.

The predominant exchangeable cation of the various bentonites was determined by the Mehlich method (5) for exchangeable cations in soils modified by Barr and Guth (4). The results are shown in Table I.

TABLE I.—THE PREDOMINATE EXCHANGEABLE CATION OF THE VARIOUS BENTONITES

	Milliequivalents per 100 Gm. Bentonite				
	H^+	Ca^{++}	Mg^{++}	K^+	Na^+
Volclay	5.6	40.6	9.8	1.0	34.8
Hydrogen	68.0	...	...	...	...
Calcium	...	79.2	...	...	...
Magnesium	...	...	63.3	...	...
Potassium	...	...	...	66.1	...
Sodium	...	...	...	...	51.3

Preparation of the Antibiotic Solutions and the Antibiotic-Bentonite Suspensions.—The required weight of the bentonite was sprinkled in divided portions on approximately 50 cc. of distilled water. When the bentonite was wetted, it was stirred with an electric mixer[7] at a moderate rate of speed until a homogenous mixture was obtained. After mixing, the spindle was rinsed with portions of distilled water and the contents transferred into a 100-cc. volumetric flask, the beaker rinsed with portions of distilled water and the rinsings added to the flask. The bentonite suspension was allowed to stand twenty-four hours to complete hydration. The contents of the flask were sterilized by autoclaving fifteen minutes at 15 pounds pressure (121°). After cooling, an aliquot of antibiotic solution, containing the desired weight of antibiotic, was added to the volumetric flask and made up to volume with sterile distilled water. The various solutions and suspensions were stored at 5° and 25° taking samples at twenty-four hour intervals and testing for antibacterial activity. The pH values of the bentonite preparations were lowered on addition of the antibiotics, but remained practically constant throughout one-hundred sixty-eight hours of storage.

Preparation of the Bacterial Culture and Test Plates.—The stock culture of *Micrococcus pyogenes* var. *aureus* (ATCC6538) was obtained from The Ohio State University, Department of Bacteriology, having been kept on nutrient agar slants. The culture was transferred to Bacto Nutrient Agar 1.5 per cent (Difco) slants, incubated at 37°, for twenty-four hours and maintained as a stock culture

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Abstracted from a dissertation submitted by August G. Danti to the Graduate School in partial fulfillment of the requirements of the degree of Doctor of Philosophy.
† Fellow of the American Foundation for Pharmaceutical Education. Present address: Assistant Professor of Pharmacy, Wayne State University College of Pharmacy, Detroit 2, Mich.
‡ Professor of Pharmacy, The Ohio State University.
[1] American Colloid Co.
[2] Surfacaine—Eli Lilly and Co.
[3] Mycifradin Sulfate—Upjohn Drug Co.
[4] Terramycin HCl—Chas. Pfizer & Co.
[5] Tetracyn HCl—Chas. Pfizer & Co.
[6] Available in various grades from the American Colloid Co.
[7] Ivan Sorwall Inc., Serial No. 10.

TABLE II.—THE RELEASE OF ANTIBIOTICS FROM SUSPENSIONS OF VARIOUS CATION-SATURATED BENTONITES[a]

Bentonites[c] 1.0 per cent w/v	Zones of Inhibition (Diam. mm.)[b] Neomycin Sulfate 250 mcg. per cc.	Neomycin Sulfate 500 mcg. per cc.	Oxytetracycline HCl 250 mcg. per cc.	Oxytetracycline HCl 500 mcg. per cc.	Tetracycline HCl 250 mcg. per cc.	Tetracycline HCl 500 mcg. per cc.
.....	26.0	28.0	38.0	39.0	39.0	40.0
Volclay	sl[d]	17.0	30.0	32.0	27.0	29.0
Na	17.0	19.0	32.0	35.0	32.0	34.0
K	17.0	18.8	34.0	36.0	34.0	35.0
Mg	NI[e]	NI	26.0	29.0	26.0	28.0
Ca	NI	NI	30.0	32.0	25.0	27.0
H	NI	NI	29.8	31.0	25.0	27.0

[a] Samples used after 24 and 168 hours of storage at 5°.
[b] Agar cup—15 mm. in diam. *Micrococcus pyogenes* var. *aureus* (ATCC6538). Incubation at 37° for twenty-four hours.
[c] All bentonite suspension controls—no inhibition.
[d] Slight, less than 17.0 mm.
[e] NI—no inhibition.

at 4°, subculturing every three weeks. From this stock culture the organism was inoculated into tubes of nutrient broth (Difco) to which was added 0.8 per cent NaCl, and incubated at 37° for twenty-four hours. Transfers were made daily in this broth for two days each time before use as an inoculum. The broth culture contained approximately 500 million organisms per cc.

The plates were prepared using the agar medium mentioned above. Thirty-one grams of the agar was suspended in 1,000 cc. of cold distilled water and then heated to boiling to dissolve the medium completely. Exactly 20 cc. of the dissolved agar was pipetted into test tubes and autoclaved for fifteen minutes at 15 pounds pressure (121°). The final pH of the medium after autoclaving was 7.2. The media from each test tube was poured into flat bottom petri dishes (15 x 100 mm.) and allowed to solidify. The plates were then inoculated with the organism using sterile cotton swabs which were immersed in the nutrient broth culture. Cups were then cut in the agar plates with a sterile cork borer having a diameter of exactly 15 mm. The plugs of agar were removed with a sterile microspatula and the bottoms of the cups were sealed with two drops of the same melted agar. After the agar had congealed, exactly 0.2 cc. of the test substance was pipetted from a sterile 1.0 cc. (tip delivery) pipet into the cup. The plates were then inoculated at 37° for twenty-four hours, after which time the zones of inhibition were measured. A Quebec colony counter and a millimeter rule were used to measure the zones. The diameter of the zone of inhibition plus the cup was recorded in millimeters. See Tables II and III.

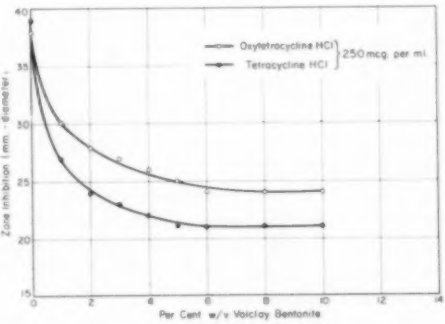

Fig. 1.—Release of oxytetracycline HCl and tetracycline HCl from various percentage w/v suspensions of Volclay bentonite.

The results of storing the preparations at 25°, as compared to 5°, were of the same order for neomycin sulfate-bentonite suspension. However, a slight decrease in the zone of inhibition was noted with both the oxytetracycline hydrochloride-bentonite and tetracycline hydrochloride-bentonite suspensions after ninety-six hours of storage. The most pronounced decrease (5 mm.) was shown with

TABLE III.—THE RELEASE OF ANTIBIOTICS FROM SUSPENSIONS OF VARIOUS CATION-SATURATED BENTONITES[a]

Bentonites[c] 2.0 per cent w/v	Zones of Inhibition (Diam. mm.)[b] Neomycin Sulfate 250 mcg. per cc.	Oxytetracycline HCl 250 mcg. per cc.	Tetracycline HCl 250 mcg. per cc.
.....	26.0	38.0	39.0
Volclay	NI	28.0	24.0
Na	sl	31.0	29.0
K	sl[d]	32.0	31.0
Mg	NI[e]	23.0	24.0
Ca	NI	25.0	21.0
H	NI	25.0	22.0

[a] Samples used after 24 and 168 hours of storage at 5°.
[b] Agar cup—15 mm. in diam. *Micrococcus pyogenes* var. *aureus* (ATCC6538). Incubation at 37° for twenty-four hours.
[c] All bentonite suspension controls—no inhibition.
[d] Slight, less than 17.0 mm.
[e] NI—no inhibition.

the antibiotic-potassium bentonite suspension after one-hundred sixty-eight hours of storage.

The Release of the Antibiotics in Various Concentrations of Volclay Bentonite.—Figure 1. Neomycin sulfate in 3 to 10 per cent w/v Volclay bentonite suspensions did not show any zones of inhibition.

The Effect of Adjusting the pH Value of Volclay Bentonite on the Release of the Antibiotics.—The antibiotic-Volclay bentonite suspensions were prepared as previously outlined. The pH value was adjusted with standard HCl (0.05 *N*) or standard NaOH (0.05 *N*), as required, after the addition of the antibiotics and made up to 100 cc. with sterile distilled water in a volumetric flask. The pH value was determined after five minutes due to fluctuations caused by the nature of the bentonite itself (Table IV).

TABLE IV.—THE RELEASE OF THE ANTIBIOTICS FROM VOLCLAY BENTONITE SUSPENSIONS AT VARIOUS pH VALUES[a]

Test Substance[c]	pH	Zone of Inhibition (Diam. mm.)[b] Hours Stored at 5° 0 Hr.	24 Hr.
NS soln.	5.90	26.3	26.3
NS in Volclay	4.00	sl[d]	sl
NS in Volclay	6.00	sl	sl
NS in Volclay	8.00	sl	sl
NS in Volclay	8.70[e]	sl	sl
OHCl soln.	3.70	37.8	38.0
OHCl in Volclay	4.00	29.0	29.0
OHCl in Volclay	6.00	29.3	29.3
OHCl in Volclay	8.00	29.8	29.8
OHCl in Volclay	7.80[e]	30.0	30.0
THCl soln.	3.70	38.8	39.0
THCl in Volclay	4.00	26.0	26.0
THCl in Volclay	6.00	25.8	26.0
THCl in Volclay	8.00	27.0	27.0
THCl in Volclay	7.80[e]	27.0	27.0

[a] 1.0 Per cent w/v Volclay bentonite.
[b] Agar cup—15 mm. in diam., *Micrococcus pyogenes* var. *aureus* (ATCC6538), incubation 37° for twenty-four hours.
[c] 250 mcg. per cc. of antibiotic.
[d] Slight, less than 17 mm.
[e] pH not adjusted.

The Effect of Ca and Mg Ions on the Release of the Antiobiotics from Sodium Bentonite Suspension.—The source of Ca ion was anhydrous $CaCl_2$ dried to a constant weight. The source of Mg ion was $MgSO_4.7H_2O$ dried at 250° for four hours to remove the last molecule of water. The antibiotic-sodium bentonite suspension was prepared as previously outlined, then a solution of $CaCl_2$ or $MgSO_4$, equivalent to 0.79 milliequivalents of Ca ion or 0.63 milliequivalents of Mg ion respectively, was added and made up to 100 cc. with sterile distilled water in a volumetric flask. The weight of Mg or Ca ion was equivalent to the number of milliequivalents in one gram of their respective bentonites. The results are shown in Table V.

DISCUSSION

The results obtained from testing the release of the neomycin sulfate could indicate a possible inactivation of the neomycin sulfate as a result of an ionic incompatibility with the bentonite. This possibility was shown by Nakashima (6) who observed an immediate coagulation or precipitation when organic cations were combined with anionic suspending agents.

Since there was a release of oxytetracycline hydrochloride and tetracycline hydrochloride from the various bentonites (Table II and III), it appeared that inactivation from a possible anionic-cationic reaction was not the entire factor. Perhaps these two antibiotics may have undergone cationic exchange with the bentonites. It has been established by investigators (7, 8) that the cations of bentonite may be replaced by organic cations as well as inorganic cations. The exchange reaction takes place practically as soon as contact is established between the clay and a solution containing the second cation. Perhaps it is the degree of cationic exchange that accounts for the difference in the amount of release of the antibiotics. If a difference does occur in the degree of cation exchange, it may be due to the differences in the activities of the cations present in the bentonites.

When dispersed in water, bentonite separates into extremely fine flakes yielding a large surface area, and the mobility of the flakes when in suspension brings them into close contact with other dissolved and suspended substances. In view of this it is possible also that the antibiotics may be adsorbed onto the surface of these particles resulting in decrease in zone of inhibition produced by the antibiotic-bentonite suspensions. From the curves shown in Fig. 1. it appears that adsorption may

TABLE V.—THE EFFECT OF Ca AND Mg IONS ON THE RELEASE OF THE ANTIBIOTICS FROM A SODIUM BENTONITE SUSPENSION[a]

Test Substance[c]	Zone of Inhibition (Diam. mm.)[b] Hours Stored at 5° 0 Hr.	24 Hr.
NS soln.	26.3	26.3
NS + Ca ion[d]	26.3	26.0
NS + Mg ion[e]	26.3	26.3
NS + Ca ion + Na bentonite	NI[f]	NI
NS + Mg ion + Na bentonite	NI	NI
NS + Na bentonite	17.3	17.0
OHCl soln.	37.8	37.8
OHCl + Ca ion	37.3	37.0
OHCl + Mg ion	36.8	37.0
OHCl + Ca ion + Na bentonite	28.8	28.8
OHCl + Mg ion + Na bentonite	28.0	28.0
OHCl + Na bentonite	32.0	32.0
THCl soln.	38.8	39.0
THCl + Ca ion	37.8	37.8
THCl + Mg ion	37.3	37.0
THCl + Ca ion + Na bentonite	24.0	24.0
THCl + Mg ion + Na bentonite	26.0	25.8
THCl + Na bentonite	32.0	32.0

[a] 1.0 Per cent w/v Na bentonite.
[b] Agar cup—15 mm. in diam. *Micrococcus pyogenes* var. *aureus* (ATCC6538), incubation 37° for twenty-four hours.
[c] Antibiotic, 250 mcg. per cc.
[d] 0.79 meq. Ca^{++} ion per cc. as $CaCl_2$.
[e] 0.63 meq. Mg^{++} ion per cc. as $MgSO_4$.
[f] NI = no inhibition.

have been a factor in the release of the antibiotic from the Volclay bentonite suspensions.

Upon addition of an acid to bentonite suspensions, cation exchange occurs between the available hydrogen ions of the added acid solution and the exchangeable cations of the bentonite. This results in a pH fluctuation. The pH values of the antibiotic-bentonite suspensions were determined when the equilibrium was established in the system, which was denoted by cessation of pH fluctuation. It appeared (Table IV) that the concentration of acid added to the antibiotic-Volclay bentonite suspensions to obtain the various pH values was insufficient to change the nature of the Volclay bentonite to the degree of performing as a hydrogen bentonite.

If equal amounts of a clay, saturated with some base, are treated with any given concentration of a chloride solution of either Na, K, Mg, Ca, or H, amounts of the base which have become replaced are found to stand in the order of Na < K < Mg < Ca < H (9). The Ca or Mg ion added to the sodium bentonite-antibiotic suspension (Table V) may have replaced the Na ion, thereby demonstrating the characteristics of a calcium or magnesium bentonite in its release of the antibiotic. The fact that oxytetracycline forms complexes with the Ca ion may prove to be a factor in the release of the antibiotic (10).

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Intracerebral Injection of Neostigmine and Eserine in Conscious Mice*

By THOMAS J. HALEY†

Intracerebral injection of 0.5–1.0 μg. of neostigmine or eserine in the conscious mouse causes the following effects: tachypnea followed by bradypnea, piloerection, lacrimation, salivation, exophthalmos, micturition, defecation, muscle spasms and tremors, loss of control of the fore limbs and generalized depression. The central sites of action producing these peripheral effects are discussed.

FELDBERG AND SHERWOOD (1) observed scratching; changes in stance, gait and posture; an alteration of awareness and stupor with signs of catatonia in conscious cats receiving intraventricular injections of eserine and diisopropyl fluorophosphonate. Inasmuch as it had been possible to duplicate Feldberg and Sherwood's (2) early work on cats using intracerebral injections in conscious mice (3), experiments were initiated to determine the type of response that could be obtained with the cholinesterase inhibitor, neostigmine, and compare it with eserine.

EXPERIMENTAL

Fifty adult mice, weighing 20–25 Gm., were restrained and direct intracerebral injections were made according to the technique described previously (3). Neostigmine methyl bromine or eserine sulfate in 0.9% sodium chloride solution was injected in a total volume of 0.05 ml. All doses refer to the salts. Control animals received a similar injection of 0.9 per cent sodium chloride solution.

RESULTS

Immediately after intracerebral injection of 0.5 μg. of neostigmine, the respiration accelerated, then slowed. There was piloerection, lacrimation, micturition, and defecation. Generalized muscle spasms and tremors with alternating loss of control of forelegs, washing of the face and scratching followed. A state of depression lasting one to two hours was observed next. When the dose was increased to 1 μg. these additional effects were noted: a "question mark" tail, salivation, and exophthalmos.

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† Chief of the Division of Pharmacology and Toxicology, Atomic Energy Project, University of California at Los Angeles.

Muscle spasms of the extensor variety occurred while the forelegs were flaccid and extended rearward under the animal (Fig. 1). At other times the mice would sit up with forelegs hanging free, the hind legs were spread widely apart to maintain balance and the head and neck were hunched over. At this time the animals had extensive muscle tremors. When the animals relaxed after such episodes, they rested part of their body weight on the first joint of the forelegs with their paws bent backward (Fig. 2). Death occurred within six minutes in 80% of the animals receiving the high dose; all the other animals survived and showed no external evidence of damage 24 hours later.

Fig. 1.—Mouse after 0.5 μg. of neostigmine, intracerebrally. Note position of forelegs in relation to hind legs.

The results with eserine were identical with those obtained with neostigmine, but in addition, there were running movements and alternating clonic and tonic seizures at the 1-μg. dose. When the animals assumed the hunched-over position, they crossed their forelegs and upon relaxation rested on the plantar surface of their feet rather than on the first joint. Twenty per cent of each group died, but the survivors showed no residual effects 24 hours later.

None of the above effects were observed in control animals receiving the saline injection. Immediately after injection the animals remained quiet for one minute then resumed their pre-injection activity. There were no residual or detrimental effects from the procedure.

DISCUSSION

The effects observed in mice after intracerebral injection of neostigmine and eserine were almost identical with those reported by Feldberg and Sherwood (1) for cats receiving eserine or diisopropyl fluorophosphonate. Whether itching actually occurs in the mouse is difficult to ascertain, but after the intracerebral injection the animals did scratch themselves in the same manner that they do when irritated locally on the skin. The action certainly was centrally induced because intravenous doses five times larger did not produce this effect. Koenigstein (4, 5) showed that intracisternal eserine and morphine elicit itching in animals and apparently the main site of action is in the distal part of the floor of the fourth ventricle. The changes in motor activity and the unnatural positions assumed by the mice are quite similar to catatonia in cats observed by Bailey (6) after midbrain lesions. Defecation may be a peripheral effect resulting from motor activity of the animals, but Kuntz (7) feels that present evidence suggests a defecation center in the medulla oblongata while Sheenan (8) related such action to the pre- and supra-optic nuclear areas of the hypothalamus. Uvnas (9) has related micturition to certain areas in the hypothalamus while Grossman and Wang (10) reported that the pre-optic area, the septum pellucidum, the medial and lateral hypothalamus, the central gray matter and the area about the central tegmental fasciculus in the midbrain were involved in bladder contraction. The changes in respiration are probably due to action on the respiratory centers in the medulla oblongata. Furstenberg and Crosby (11) have related salivation to stimulation of the pre-optic hypothalamus, but the salivatory nuclei may also be involved. Fulton (12) has related piloerection to stimulation of the lateral hypothalamic areas particularly the medial nuclei. However, the premotor

Fig. 2.—Effect of 0.5 μg. of neostigmine on body positioning of mouse. Animal rests on first joint of forelegs.

area of the cerebral cortex may also be involved in piloerection (Kuntz, 13). Lacrimation has been related to area 8 of the cerebral cortex (Bechterew and Mislawski, 14). Such effects on the premotor cortex may be brought about by stimulation of the parasympathetic centers in the anterior hypothalamus. Furthermore, the lacrimal gland may be activated by impulses from the mammillary body to the nucleus of the facial nerve in the brain stem. Alexander (15) has related tremors to effects on the globus pallidus and substantia nigra with impulses going to area 4 of the cerebral cortex. However, the dentate nucleus as well as the lobulus ansiformis of the cerebellum could be involved in the tremor produced by neostigmine and eserine. Waller (16) has shown that the locomotor point for running movements is in Forel's field at the level of the subthalamic decussation, dorsal to the mammillary body near the subthalamic body of Luys. This area could be reached by the drugs and account for the running movements seen.

All of the above possible sites of central activity of neostigmine and eserine could be reached by the

drug after intracerebral injection because the technique used allows introduction into the ventricular system as well as access to the subarachnoid space via the foramina. Intraneuronal connections must also play a part in activating centers remote from the site of injection.

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Stabilization of Ethiodol Emulsion for Intravenous Administration*

By F. S. HOM,† J. AUTIAN, A. N. MARTIN, J. EDWARD BERK, and J. GEORGE TEPLICK

A study was conducted to prepare a stable Ethiodol emulsion for intravenous administration which may be utilized as a contrast medium to visualize the liver and spleen. Because Ethiodol is an oil-like substance having a specific gravity greater than water, the stabilization problem is more acute. A number of emulsifiers and stabilizing agents were utilized in order to prepare an o/w emulsion which would remain stable for a reasonable period of time and possess the particle size necessary for intravenous administration. Using polyoxyethylene sorbitan monooleate[1] and sorbitan monooleate[1] a stable emulsion was formed with the aid of a two-stage laboratory homogenizer.

THE SUCCESSFUL APPLICATION of Ethiodol[2] by Palmer (1) for use in hysterosalpingography has created interest in this contrast medium for other diagnostic studies such as hepatolienography. Teplick and Berk (2) have suggested an emulsion form of Ethiodol to be administered intravenously for the radiopaque visualization of the liver and spleen.

Ethiodol is an ethyl ester of iodinated fatty acid of poppyseed oil containing 37 per cent organically bound iodine. The oil is amber-colored, is extremely fluid, and has a specific gravity of 1.28.

Oils for intravenous administration present certain problems. It is desirable to use an o/w emulsion for such materials. The emulsion must be prepared in such a manner that a free flowing product having a mean particle size of less than four microns is produced (3). The particle size distribution must not change during storage and the finished product must be sterile and compatible with blood.

This project is divided into three parts covering the pharmaceutical aspects, the toxicity studies, and clinical trials. This paper is limited to the pharmaceutical aspects of the problem. The toxicity studies and clinical trials will be published elsewhere.

EXPERIMENTAL

The Use of Sorbitan Esters as Emulsifying Agents.—Griffin's (4) method of assigning HLB values to surface-active agents was used to deter-

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† Fellow of the Sidney Hillman Foundation.
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[1] Products of Atlas Powder Co., Wilmington, Del. Tween® 80—polyoxyethylene sorbitan monooleate; Span® 80—sorbitan monooleate.
[2] Trademark name of the E. Fougera & Co., Inc., New York, N. Y. for a very fluid contrast medium. It differs from "Lipiodol" (brand name by Fougera for Iodized Oil, U. S. P.) in that the Ethiodol is the ethyl ester of iodinated fatty acid of poppyseed oil. Ethiodol differs from iodized oil by its extreme fluidity as compared to the very viscous nature of the iodized oil. For further clarification on nomenclature the reader is referred to reference (1).

mine the proper blend of Spans and Tweens. Emulsions were prepared using 50 per cent[3] of oil and 5 per cent of total emulsifiers. Ingredients were weighed directly into a tared, wide mouth, four ounce, screw capped bottle. The distilled water was measured from a cylindrical graduate and added to the oil previously combined with the emulsifying agents. After the cap was secured, the mixture was vigorously agitated for several minutes. Tables I, II, and III show the results of using several combinations of Spans and Tweens. Comparison of three sets of experiments over a one-week period reveals that the combination of Span 80 and Tween 80 produced superior emulsions. Results were based upon rate of creaming, dispersibility, and general physical appearance.

A second series of experiments were conducted as above except that cross-combinations of Spans and Tweens were utilized to give an HLB of 12.5 in each sample. As observed in Table IV, the combination producing the best emulsion was Span 80 and Tween 80.

TABLE I.—HLB DETERMINATION WITH SPAN 60 AND TWEEN 60

Sample	Span 60, %	Tween 60, %	HLB	Result
1	100	0	4.7	Cracked
2	75	25	7.25	Poor
3	50	50	9.80	Good
4	25	75	12.35	Good
5	0	100	14.35	Good

TABLE II.—HLB DETERMINATION WITH SPAN 20 AND TWEEN 20

Sample	Span 20, %	Tween 20, %	HLB	Result
1	80	20	10.22	Poor
2	60	40	11.84	Fair
3	50	50	12.65	Good
4	40	60	13.46	Fair
5	20	80	15.08	Poor

TABLE III.—HLB DETERMINATION WITH SPAN 80 AND TWEEN 80

Sample	Span 80, %	Tween 80, %	HLB	Result
1	25	75	12.33	Excellent
2	20	80	12.86	Excellent
3	15	85	13.40	Good
4	10	90	13.93	Fair
5	5	95	14.47	Poor

Aseptic Preparation of the Emulsion and Determination of Optimum Concentration of Span 80 and Tween 80.—Emulsions of 50 per cent oil-in-water were formulated and prepared with total emulsifier of 3, 4, 5, 6, 7, and 9 per cent. The emulsions were adjusted to a pH of 7.4 with mono- and dibasic sodium phosphate and preserved with a combination of methylparaben and propylparaben. The emulsions were adjusted to approximate the same osmotic pressure as that of the blood using dextrose. Isotonicity calculations were based on the water-phase concentration. The emulsions were prepared as follows:

[3] All percentages are calculated upon a weight basis.

TABLE IV.—SIMPLE CROSS-COMBINATION OF TWEEN AND SPAN (HLB 12.5)

Sample	Span	%	Tween	%	Result
1	40	41	20	59	Fair
2	60	34	20	66	Fair
3	80	32	20	68	Fair
4	20	43	40	57	Good
5	60	27	40	73	Fair
6	80	22	40	78	Fair
7	20	35	60	65	Good
8	40	27	60	73	Fair
9	80	21	60	79	Good
10	20	37	80	63	Good
11	40	29	80	71	Fair
11	60	23	80	77	Fair
13	80	25	80	75	Excellent

Span 80 and Ethiodol were weighed into a tared, sterile beaker. The solution was poured into a Waring Blendor which had previously been washed and rinsed with sterile distilled water. The Tween 80 was weighed in a tared, sterile beaker to which was added an aqueous solution of the buffer and preservatives. The resulting mixture was stirred and added gradually in small portions to the oil phase in the blendor. After five minutes of agitation, the primary emulsion was poured into the hopper of a two-stage Gaulin Laboratory Homogenizer[4] which had been washed and rinsed previously with water for injection. The emulsion was then recycled through the homogenizer for a thirty-minute period. The pressure gauge was adjusted to 3000 p. s. i. for the first stage and 500 lbs. for the second stage. A temperature of 75° or higher was recorded for the emulsion during the recycling process.

At the end of this period, the emulsion was collected in sterile Erlenmeyer flasks, was stoppered and allowed to cool to room temperature. The emulsion was filtered through a medium porosity sintered glass filter under reduced pressure. Samples of the emulsion were taken at this point to determine the pH and particle size. The pH values were usually about 7.5. Particle size was determined under a microscope fitted with an optical micrometer. Average particle size was less than one micron, while in no instance was the particle size greater than three microns. The emulsions were finally placed in 50-ml. multiple dose vials and intermittently sterilized by placing them in a 60° hot-air oven for two hours each day for three successive days.[5] Autoclaving was too severe for these emulsions since it resulted in a complete breakdown of the emulsion. It was found that 6 per cent was the optimum total concentration of emulsifiers to produce a stable emulsion. The 6 per cent emulsion was stable to freezing and thawing, to 0.5 *N* HCl

[4] Laboratory Homogenizer with two horsepower motor, Manton-Gaulin Mfg., Inc., Everett, Mass.

[5] Sterility tests on the emulsions will be reported in a later publication.

and 0.5 *N* NaOH and to dilution with water. It also was compatible with blood serum *in vitro*.

The Use of Gelatin and Asolectin as Emulsifying Agents.—A 50 per cent emulsion was prepared in a two-stage homogenizer using a one and three per cent concentration of gelatin[6] as the emulsifying agent. Both emulsions were excellent, but on standing the first emulsion congealed when stored in a cool place; the three per cent emulsion congealed at room temperature.

Another emulsion was prepared as above using three per cent Asolectin[7] as the emulsifying agent. This emulsifier gave very poor results.

Stability Study of Ethiodol Emulsion.—From a number of exploratory formulas and the study described here, Formula *A* was developed:

Formula A	Gm.
Sterile Ethiodol	900.00
Tween 80	90.00
Span 80	30.00
Na_2HPO_4 (anhydrous)	6.76
$NaH_2PO_4 \cdot H_2O$	0.74
Methylparaben	3.60
Propylparaben	0.40
Water for injection	968.50
to make about	2000.00

The emulsion was prepared as described previously utilizing the two-stage homogenizer. It proved stable to dilute HCl and NaOH solutions and to freezing and thawing. The emulsion was compatible with blood serum *in vitro* and it was approximately iso-osmotic with the blood as determined by the cryoscopic method.

Several samples were stored at various temperatures and were observed for a period of eight weeks. The storage temperatures were 5°, 25°, 40°, and 60°. After eight weeks, all but the sample stored at 60° proved stable. This emulsion had broken and the pH had dropped from 7.4 to 2.3. The evaluation of stability was based upon the following features: rate of creaming, dispersibility, particle size, pH, and general physical appearance. The average particle size was approximately one micron with no particles greater than three microns.

Emulsion samples were placed into 50-ml. burets, stoppered, and kept at room temperature for six months. There were no signs of sedimentation at the end of this time.

DISCUSSION

Although the HLB (hydrophil-lipophil balance) is quite important for the formulation of a stable emulsion, the chemical type of the emulsifying agent must also be considered (4). For example, it can be seen from Tables I to III that although the HLB of the best emulsion in each series was approximately 12.5, Span 80 and Tween 80 produced the superior emulsion. Further proof may be gleaned from Table IV. Here a number of cross-combinations of Spans and Tweens adjusted to an HLB of 12.5, once again indicating that Span 80 and Tween 80 were the better combination. It would thus seem that the lipophil portion of Span 80 and Tween 80 is more closely related to the chemical nature of the components of the Ethiodol than the other Spans and Tweens. The chemical structures of the Span 80, Tween 80, and the oil suggest that this assumption is reasonable.

Meticulous care must be exercised in formulating an intravenous emulsion since particle size larger than two microns may cause embolic phenomena. A great deal of energy must be supplied to "break" the oil phase into the small particles. In this study, the energy was supplied mainly by the use of a two-stage pressure homogenizer. Five hundred pounds of pressure exerted on the second stage was employed to prevent agglomeration. The preparation of the primary emulsion in the Waring Blendor assures uniform mixing and quicker homogenization.

As the particle size in an emulsion decreased, the surface area of the oil phase is increased greatly which necessitates an increased amount of emulsifying agent. In this investigation, concentration of emulsifying agents was 6 per cent.

Gelatin (5) and Asolectin (6) have both been used as emulsifying agents for intravenous emulsion; however, in this study they did not produce a satisfactory product. The gelatin emulsion had a tendency to congeal and to agglomerate while Asolectin produced a coarse emulsion.

Emulsions for intravenous injection must be prepared under aseptic conditions and sterilized after manufacture. Intermittent sterilization at 60° was found to be necessary since higher temperatures degradated the emulsion. The storage at various temperatures indicated the excellent stability of this emulsion, and further proof of stability was shown by the sedimentation study. Storage at 60° decreased the stability of the emulsion.

The emulsion was weakly buffered to pH 7.4, the pH value of the blood. Cryoscopic study indicated that the emulsion was approximately isotonic with the blood. Preliminary investigation with animals has indicated the usefulness of this emulsion to visualize the spleen and liver on intravenous administration by roentographic methods.

SUMMARY AND CONCLUSION

The use of an emulsion containing a radiopaque contrast medium to be injected intravenously is not a new concept, but the preparation of such a product which is both stable and can be injected into the vein has not met with great success. This paper suggests a method for preparing such a product. The experimental regimen which was employed included the following stages:

1. The optimum Tween-Span combination for the preparation of a stable Ethiodol emulsion was determined by the HLB method of Griffin (4). A total of 6 per cent of Tween 80 and Span 80 in a ratio necessary to yield an HLB 12.5 was found to produce the most stable product.

2. Gelatin and Asolectin were found to be unsuitable as emulsifying agents for this product.

3. The stability of the emulsions was estimated by gross and microscopic observation of

[6] Product obtained as i. v. 6% Gelatin Solution, Charles B. Knox Gelatine Co., Camden, N. J.

[7] Product obtained from Associated Concentrates, Woodside, N. Y.

samples stored at room temperature and at an elevated temperature.

4. Based on the above finding, a stable emulsion of a radiopaque contrast medium for intravenous injection was formulated. The method of preparation and sterilization were discussed. Pharmacological and clinical studies on this emulsion will be reported at a later time.

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The Effect of Type and Concentration of Binders on the Hardness and Compressibility of Sulfathiazole Tablets Made at Constant Pressure*

By EINO NELSON, JACK R. ARNDT, and L. W. BUSSE

A number of sugars, gums, and miscellaneous substances were evaluated as binders in sulfathiazole tablets by means of hardness determinations and compressibility studies. It was found possible to rank binders in order of hardness imparted to tablets as well as to classify them functionally as exhibiting sugar-like or gum-like behavior. Binders possessing sugar-like behavior showed maxima in hardness *versus* concentration curves. The maxima usually occurred at a binder concentration of 4% or 8%. Increasing the concentration of gum-like binders gave increasingly hard tablets but the percentage increase in hardness decreased. Fair correlation was observed between compressibility of a granulation and the hardness of tablets resulting from its compression.

NO INFORMATION is presently available giving quantitative data on the effect of type or concentration of binder on hardness of tablets. This is probably due to the fact that until relatively recently there was no way available to make tablets for testing under controlled and reproducible compression pressures (1). Since pressure and binder concentration both contribute to final tablet hardness for a given granulation system, their individual contributions are not separable until pressure can be controlled, measured, and reproduced for successive tablets.

This work was performed to determine the effect of a number of sugars, natural and synthetic gums, and other substances either used or appearing to have potential as binders on the hardness and compressibility of sulfathiazole tablets made under isobaric conditions. The effect of binder concentration was studied concurrently.

* Received August 16, 1956, from the School of Pharmacy, University of California, San Francisco, and the School of Pharmacy, University of Wisconsin, Madison.

EXPERIMENTAL

Test Granulations.—Granulations were made 1, 2, 4, 8, 12, and sometimes 100% with respect to most of the binders that exhibited behavior classified as sugar-like and usually 0.1, 0.2, 0.4, 1.0, 2, and 4% with respect to the other materials studied. The source and purity of binders used are listed in Table I. Those sugars that contained water of hydration in their usual state were used in test granulations in an amount sufficient to give the indicated percentages on an anhydrous basis.

Granulations were made in 50- or 100-gram lots. An appropriate amount of sulfathiazole powder was weighed out and to this was added a sufficient amount of binder in solution to give the desired percentage of binder in the granulation on a dry basis. The wet mixtures obtained by this procedure were forced through a 20-mesh sieve and placed in a hot air oven to dry at about 45 degrees for 4 to 8 hours. At the higher binder concentrations, it sometimes happened that the mixture was too wet to granulate. In this case the mixture was allowed to air dry until its consistency was such as to allow granulation by the sieve procedure. The moisture content of dried granulations was determined with a Cenco Moisture Balance. Moisture content was between 0.1 and 0.4% for all granulations.

TABLE I.—SUBSTANCES USED AS BINDERS

Compound	Source	Purity
Tragacanth	.....	.....
Pectin	California Fruit Growers' Exchange, Ontario, California	Pure
Carrageenin binder	Algin Corporation of America	Not specified
Mannitol	Atlas Powder Co., Wilmington, Del.	Not specified
Gelatin	Entreprises Chimiques et Électriques Vilvoide, Belgium	Not specified
Gelloid 50	Jacques Wolf and Co., Passaic, N. J.	Not specified
Carboxymethylcellulose	Hercules Powder Co., Wilmington, Del.	Type: 70 Premium (medium viscosity)
Methylcellulose	The Dow Chemical Co., Midland, Mich.	Pharmaceutical grade. 100 c. p. s.
White dextrin	Merck and Co., Inc., Rahway, N. J.	N. F. V
L(+)Arabinose	Pfanstiehl Laboratories, Inc.	C. P.
Dextrose	Baker and Adamson	Reagent
Fructose	Eastman Kodak Co.	Not specified
D(+)Galactose	Pfanstiehl Laboratories, Inc.	C. P.
L-Rhamnose	Fisher Scientific Co.	C. P.
D(+) Xylose	Pfanstiehl Laboratories, Inc.	C. P.
Sucrose	Colonial Sugar Co.	Not specified
Lactose	Mallinckrodt Chem. Works	U. S. P. powder
Maltose	Merck and Co., Inc.	Purified
Raffinose hydrate	The Matheson Co.	Not specified
Acacia	S. B. Penick and Co.	U. S. P.
Sterculia	.....	.....

After drying, the granulations were sieved to collect 20/30 mesh fractions. These samples were placed in tightly stoppered containers until weighed for experimental compressions.

Compressions.—All compressions were made to an intensity of 1,100 Kg./sq. cm. (about 16,000 p.s.i.), using a previously described instrumented tablet machine (1). All tablets were made from 380 ± 1 mg. of test granulations, using a $^{3}/_{8}$-inch flat-faced punch and die set. Prior to each compression the die wall was dusted with magnesium stearate powder to equalize upper and lower punch pressure (2). Five tablets were made from each granulation representing a single concentration of one binder. During the compressions, upper punch displacement and lower punch pressure were recorded as functions of time. The time for the compression event was about 2 seconds.

Hardness Determination.—Hardness tests were made one month after compression using a Strong Cobb hardness tester. The pump on this tester was disconnected and air under pressure from a storage tank was used to activate its mechanism. Pressure and flow were adjusted to cause the rate of change of force applied to tablets under test to be 0.85 Kg./second.

RESULTS AND DISCUSSION

Effect of Nature and Concentration of Binder on Hardness and Compressibility.—It became apparent when hardness determinations were made that two types of binder behavior had occurred. With those materials listed in Table II, a maximum occurred when hardness was plotted as a function of binder concentration. The materials in this table are designated "sugar-like binders" since most of the materials in the table are sugars. The other type behavior was characteristic of natural gums and plots of hardness *versus* concentration of binder showed a progressive increase in hardness with increasing concentration. The behavior was further characterized by a progressive decrease in percentage increase of hardness. These materials were classified as gum-like and hardness and concentration data are tabulated in Tables IIIa and IIIb.

The nature of the hardness *versus* concentration plots obtained with sucrose, dextose, and raffinose are shown in Fig. 1. These are typical for most of the materials in Table II. The maxima in hardness *versus* concentration curves occurred most often at

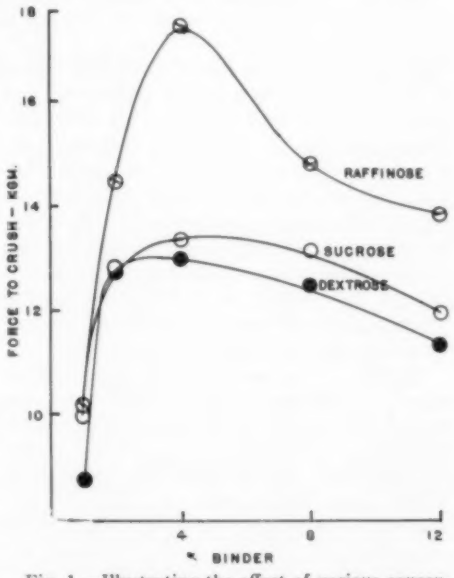

Fig. 1.—Illustrating the effect of various concentrations of sugar binders on the hardness of sulfathiazole tablets made at constant pressure.

TABLE II.—HARDNESS OF SULFATHIAZOLE TABLETS MADE WITH SUGAR-LIKE BINDERS AS FUNCTION OF BINDER CONCENTRATION

Binder	Kg. Force to Crush[a] Concentration of Binder, % 1	2	4	8	12	100
Arabinose	10.4	12.7	14.1	11.2	13.9	...
	(0.8)	(2.2)	(1.8)	(1.7)	(5.7)	...
Carboxymethylcellulose	84.0	20.3	19.8	...	...	
	(1.4)	(1.5)	(1.5)	...	...	
Dextrin	9.0	9.8	13.1	13.4	16.0	5.7
	(0.3)	(0.9)	(1.0)	(1.3)	(1.5)	(1.0)
Dextrose	8.8	12.8	13.0	12.5	11.3	4.1
	(0.8)	(1.4)	(1.9)	(1.7)	(1.5)	(0.9)
Fructose	6.3	6.2	5.9	5.4	5.4	5.7
	(0.8)	(0.8)	(0.5)	(0.2)	(2.0)	(0.6)
Lactose	7.6	9.1	9.8	9.5	8.7	10.8
	(0.9)	(0.8)	(1.6)	(1.1)	(1.0)	(3.0)
Mannitol	7.3	10.2	9.2	9.3	8.8	...
	(0.9)	(0.8)	(0.4)	(2.4)	(0.6)	...
Raffinose hydrate	10.4	14.5	17.5	14.8	13.9	12.2
	(1.5)	(1.2)	(2.0)	(2.4)	(1.8)	(0.9)
Rhamnose	9.8	12.3	12.5	14.8	13.1	...
	(2.0)	(1.8)	(1.8)	(3.1)	(2.8)	...
Sucrose	10.0	12.9	13.3	13.2	12.0	7.7
	(1.7)	(1.7)	(1.9)	(1.1)	(1.3)	(0.7)
Xylose	11.2	11.9	12.8	14.4	16.2	...
	(0.5)	(3.5)	(2.7)	(4.4)	(1.9)	...

[a] Mean of 5 determinations. Mean plus and minus quantity in brackets is its 95% confidence interval (3).

TABLE IIIA.—HARDNESS OF SULFATHIAZOLE TABLETS MADE WITH GUM-LIKE BINDERS AS FUNCTION OF BINDER CONCENTRATION

Binder	Kg. Force to Crush Tablet[a] Concentration of Binder, % 0.1	0.2	0.4	1.0	2	4
Acacia	6.5	7.0	7.8	8.8	11.0	14.2
	(1.8)	(1.4)	(0.6)	(0.4)	(0.7)	(1.7)
Carrageenin binder	6.2	6.7	8.7	12.4	13.8	17.8
	(1.2)	(0.9)	(0.6)	(0.9)	(1.0)	(1.0)
Gelatin	8.9	10.7	12.7	16.0	20.6	22.9
	(0.9)	(0.8)	(1.9)	(2.5)	(1.7)	(2.1)
Gelloid 50	8.2	8.8	9.7	13.8	19.1	21.9
	(0.4)	(1.7)	(1.5)	(0.3)	(3.0)	(0.9)
Methylcellulose	6.4	7.6	10.6	16.0	19.9	24.1
	(1.1)	(0.9)	(1.0)	(2.2)	(1.0)	(1.8)
Pectin	6.9	7.6	10.1	13.7	14.9	21.9
	(1.4)	(0.5)	(2.6)	(0.8)	(1.7)	(2.6)
Sterculia	7.3	7.1	7.5	10.9	11.6	16.3
	(1.3)	(1.2)	(1.1)	(1.3)	(0.3)	(3.0)
Tragacanth	6.4	6.1	7.5	12.5	14.8	20.1
	(0.4)	(0.4)	(1.3)	(2.1)	(2.8)	(2.3)

[a] Mean of 5 determinations. Mean plus and minus quantity in brackets is its 95% confidence interval (3).

TABLE IIIB.—HARDNESS OF SULFATHIAZOLE TABLETS MADE WITH GUM-LIKE BINDERS AS FUNCTION OF BINDER CONCENTRATION

Binder	Kg. Force to Crush Tablet[a] Concentration of Binder, % 1	2	4	8	12	100
Galactose	7.2	11.8	12.9	11.8	12.7	...
	(1.5)	(2.5)	(1.3)	(2.9)	(4.4)	...
Maltose	6.0	7.4	9.5	11.9	9.7	24.4
	(0.5)	(1.5)	(2.0)	(2.5)	(1.6)	(3.3)

[a] Mean of 5 determinations. Mean plus and minus quantity in brackets is its 95% confidence interval (3).

4%. Exact location was complicated by variation and relatively small changes in hardness.

Compressibility data obtained during the manufacture of tablets made with sugar-like binders tended to show the validity of the occurrence of maxima. The logarithm of lower punch pressure is plotted as a function of upper punch displacement in Fig. 2 for sucrose and Fig. 3 for raffinose hydrate.

These plots were constructed by reading displacement from the time of application of 280 Kg./sq. cm. pressure to the granulation until a pressure near 1,000 Kg./cm.^{2} was applied. There is a progressive decrease in slope with increasing concentration of binder until a minimum occurs which should more accurately locate the concentration at which maximum hardness was obtained in tablets. Sulfathiazole granulated with water and processed like the other test granulations yielded a curve with slope greater than any of the curves shown in Figs. 2 and 3.

The slopes of the curves of Figs. 2 and 3 are plotted *versus* concentration in Fig. 4. Sucrose passed through a minimum in slope at 8% yet examination of Fig. 1 shows that a maximum in hardness occurred at 4%. It is apparent that variation in hardness values did not allow correct location of the maximum in this case. Using the compressibility criterion to select concentration for maximum hardness yielded data for Table IV where this criterion and hardness test criterion are compared.

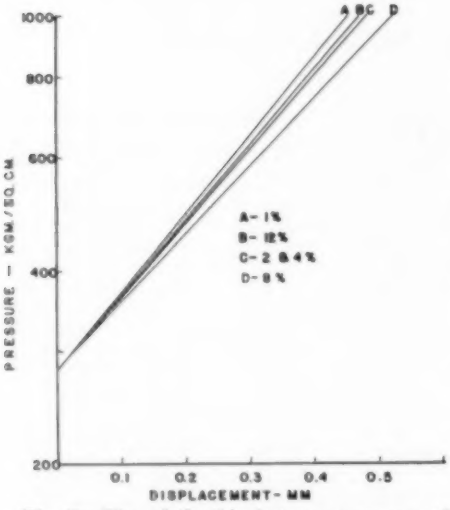

Fig. 2.—The relationship between pressure and punch displacement for the compression of sulfathiazole tablets made with several concentrations of sucrose as binder.

Many of the sugar-like materials gave compressibility curves that were nonlinear when log of pressure was plotted against punch displacement. However, the relative location of the curves for a given substance in a range of concentrations was in qualitative agreement with relative hardness on hardness-concentration plots. As was the case with sucrose and raffinose, the most compressible granulation was taken to yield the hardest tablet for entry in Table IV.

The reason for the occurrence of maxima in the function relating hardness and concentration is probably complex since no single property of material compressed or binder used determined hardness. The property of binding material to adhere to itself and the substrate with which it is mixed is a quality

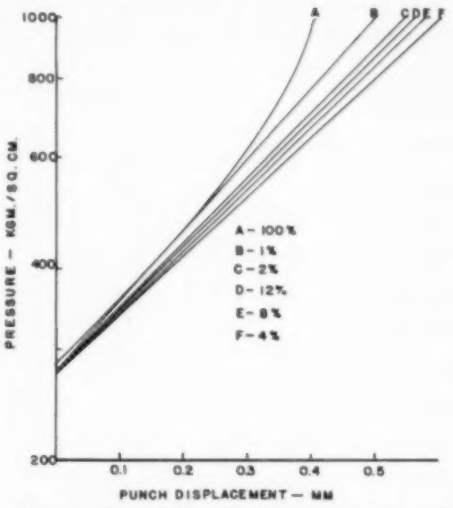

Fig. 3.—The relationship between pressure and punch displacement for the compression of sulfathiazole tablets made with several concentrations of raffinose as binder.

that is related to degree of increase in hardness with increasing pressure and the difference in hardness when several materials are compared under isobaric and isometric conditions. Yet, change in granule shape during compression related to the compressibility of both the binder and substrate also influences tablet hardness as has been shown (4). The interaction of these effects could cause the complex behavior observed.

The relation between hardness and concentration found with gum-like binders is shown in Fig. 5 for

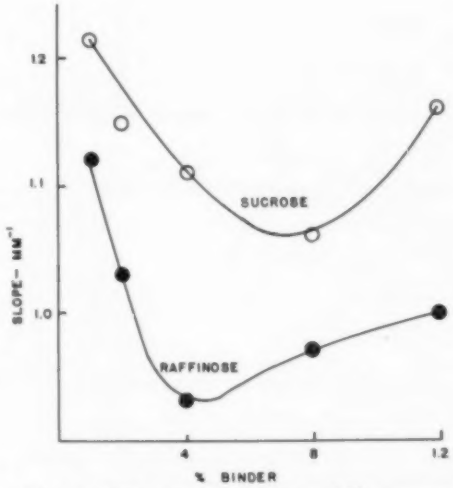

Fig. 4.—The slopes of compressibility curves plotted as a function of binder concentration for sucrose and raffinose. Data from Figs. 2 and 3.

TABLE IV.—CONCENTRATION OF SUGAR-LIKE BINDERS TO OBTAIN MAXIMUM HARDNESS IN SULFATHIAZOLE TABLETS

Binder	Concentration of Binder for Maximum Hardness, %: From Hardness Data	From Compressibility Data
Arabinose	4	8
Carboxymethylcellulose	2	1
Dextrin	12	12
Dextrose	4	8
Fructose	2	2
Lactose	4	8
Mannitol	2	4
Raffinose hydrate	4	4
Rhamnose	8	8
Sucrose	4	8
Xylose[a]	12	..

[a] Differences in compressibility data not marked enough to allow choice of a most compressible composition.

gelatin, tragacanth, and acacia. The logarithm of pressure is plotted against punch displacement for gelatin in Fig. 6 and for acacia in Fig. 7.

The behavior of the gum-like materials was more like that which would be expected in studying binder-substrate systems. The curves of hardness *versus* concentration plots were probably asymptopic to the hardness which would be found from studying pure binder in the granular form of the mixtures used in compressions, but it was not practical to test this contention. The property possessed by gum-like materials to adhere to themselves was evidently dominant in determining hardness as contrasted to the factors that may have influenced the results with sugar-like binders.

It was not possible to rectify compressibility data for all gum-like binders on log pressure *versus* punch displacement plots. However, it was again noted that the relative location of the compressibility curves was in agreement with relative location of corresponding points on hardness *versus* concentration plots.

Maltose and galactose showed maxima in hardness *versus* concentration plots which were not substantiated in compressibility studies; hence, their

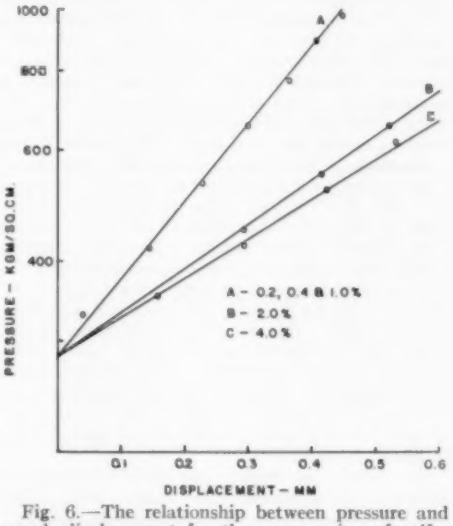

Fig. 6.—The relationship between pressure and punch displacement for the compression of sulfathiazole tablets made with several concentrations of gelatin as binder.

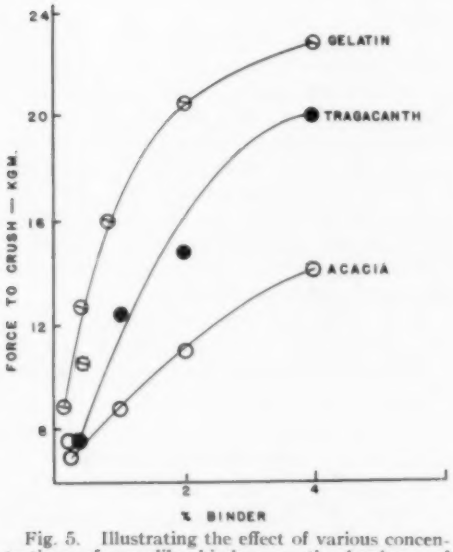

Fig. 5. Illustrating the effect of various concentrations of gum-like binders on the hardness of sulfathiazole tablets made at constant pressure.

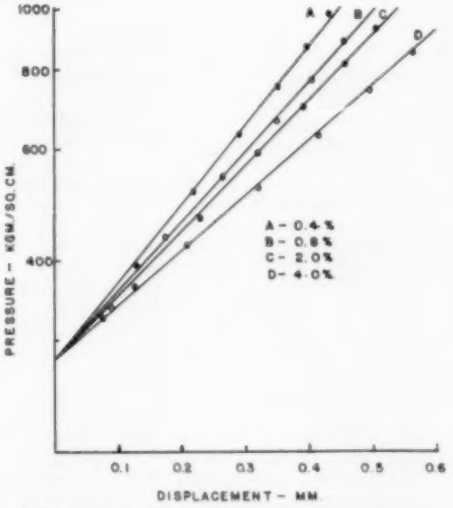

Fig. 7.—The relationship between pressure and punch displacement for the compression of sulfathiazole tablets made with several concentrations of acacia as binder.

classification as gum-like. In the case of maltose, tablets made of this material gave a mean hardness of 24.4 Kg., a value which was considerably higher than that obtained using this material on sulfathiazole to a maximum concentration of 12 per cent. This behavior contrasts sharply with hardness obtained using other sugar-like binders only to make tablets as can be seen from re-examination of Table II.

Hardness and compressibility data leave no doubt as to the existence of an optimum concentration of sugar-like binder for use. The data obtained does not rule out the possibility of the existence of such a condition for the gum-like materials. The existence does seem unlikely because of the extreme hardness obtained and the rapid rise to maximum hardness with small concentrations of binder. At the concentrations the binders studied are used in practice, the classification is certainly correct.

Ranking of Binders in Order of Hardness.—The binders used are ranked in order of hardness in Table V. This was done by first finding the mean of the hardnesses measured at concentrations of 2 and 4% binders present. Then, the material giving the hardest tablets (methylcellulose 100 c. p. s.) was assigned a binding index of 100. The binding index for the other materials was computed by multiplying the mean hardness obtained for other materials by the ratio: $100/A$, where A = mean hardness of tablets made with methylcellulose.

A classification such as this is admittedly arbitrary but does reflect to some extent at least the ability of the substances tested to impart strength to sulfathiazole tablets.

Significance of Results for Other Systems.—Whether or not qualitatively similar results could be obtained using these binders on other materials cannot be predicted with certainty. However, the results should be similar for those substances that possess a low order of binding affinity toward themselves, particularly when gum-like binders are used. This was the case with the sulfathiazole studied here which, when made into a granulation by moistening with water and further processed to make tablets at the same pressure as used for the other materials studied, yielded tablets that required a mean crushing force of 8.5 Kg.

Significance of Results in Tablet Formula Construction.—The appearance of maxima in hardness *versus* concentration curves with sugar-like binders indicates that any binder of this class should be used at the concentration where the maximum occurs or at some lower concentration. If sufficiently hard tablets do not result even at the optimum concentration and increased compression pressure increases disintegration time excessively, then a different binder should be used.

When gum-like binders are used it should be recognized that a diminishing return in terms of increase in hardness occurs at low concentrations of these substances.

In terms of final tablet properties, satisfactory disintegration time is as important as satisfactory hardness. In most instances this can be achieved by the use of sufficient quantities of starch or alginic acid as a disintegrating agent. Since the standard disintegration time test gives no information on the availability of the drug from the granules into which a tablet disintegrates, it should be recognized that drugs incorporated into tablets using the sugar-like binders will in general be more readily available than when gum-like binders are used. This is due to the influence of their greater solubility on solution rate.

TABLE V.—LISTING OF BINDERS IN ORDER OF STRENGTH IMPARTED TO SULFATHIAZOLE TABLETS

Binder	Binding Index[a]
Methylcellulose 100 c. p. s.	100
Gelatin	99
Gelloid 50	93
Carboxymethylcellulose	91
Pectin	84
Tragacanth	79
Raffinose hydrate	73
Carrageenin binder	72
Sterculia	63
Arabinose	61
Sucrose	60
Acacia	57
Xylose	56
Galactose	56
Rhamnose	56
Dextrose	52
Dextrin	52
Mannitol	44
Lactose	43
Maltose	38
Sulfathiazole (no binder)	38
Fructose	27

[a] See text for meaning and computation of index.

CONCLUSIONS

1. It was possible to study and classify binders by means of crushing tests made on tablets compressed at known and reproducible compression pressure.

2. Two types of binder behavior occurred when various substances were used as binders on sulfathiazole powder. One type of behavior was classifiable as sugar-like and the binder's behavior was characterized by a maximum hardness in the tablets occurring usually at around 4 per cent of binder present. The other behavior was classified as gum-like and the binder's behavior was characterized by a progressive decrease in percentage increase of strength with increase of binder concentration.

3. A correlation appeared to exist between the compressibility of a granulation and the hardness of tablets that resulted from its compression.

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The Absorption of Sulfur Compounds from Externally Deposited Polythionates by the Skin of the Rat*

By TORBEN E. NEESBY, ANTHONY W. PIRCIO, and JEROME F. GRATTAN

A study has been made of the absorption of sulfur compounds from topically applied S^{35}-labeled polythionates by the skin of the adult rat. The amount of S^{35} absorbed by the skin increased with the time of application. Some of the absorbed S^{35} entered the systemic circulation. The data indicate that the degree of absorption of topically applied polythionates is independent of the amount of polythionates applied on the skin over the concentration range studied. The uptake of S^{35} into the skin of the rat was also studied by means of autoradiography.

SULFUR and its compounds have been used traditionally for centuries in the treatment of dermatological disorders, especially acne and pityriasis (1).

The mechanism of action of finely divided sulfur on the skin has been discussed by Lomholt (2), Goodman and Gilman (3), and others (4, 5). It has been proposed that the oxidation products of sulfur are responsible for the fungicidal and bacteriological properties of sulfur (6, 7, 8). The two conversion products of sulfur to which the therapeutic activity of sulfur has been attributed are hydrogen sulfide and pentathionic acid (3). The latter has been shown to be both germicidal and fungicidal (9). Solutions of hydrogen sulfide, on the other hand, have relatively little effect on pathogenic organisms (2), so that it is more likely that pentathionic acid accounts for the therapeutic activity of sulfur (10, 11).

Solutions of polythionates are bactericidal in acid media only (12, 13). The keratolytic property of sulfur may be the basis for its therapeutic activity in certain skin disorders not directly associated with infection.

Although clinical confirmation of the value of diluted solutions of polythionates has recently been published (2, 10, 11, 14, 15), the mode of action of topical sulfur still remains undefined. To our knowledge a thorough investigation of the mode of action of topically applied sulfur and polythionates has never been undertaken.

Several papers have been published dealing with the penetration of sulfur (in various vehicles) through the intact skin (16–20). Moncorps (16), in 1928, observed an increase in the sulfur content of the urine twelve hours after the application of a 10 per cent precipitated sulfur ointment on the skin. More recently Stüttgen and Wüst (18) have shown that topically applied elemental sulfur in the form of a white vaseline ointment plus cholesterol (Eucerin) was absorbed through the skin of humans. Chevremont and Frederic (17) have shown that ferric ferricyanide will react with the thiol groups in sections of skin. The blue color which develops after the application of this reagent, histochemically locates the sulfur in the section of the skin. The intensity of the color produced is claimed to be dependent upon the amount of sulfur present in the skin. Unpublished experiments in our biological laboratory employing the Chevremont technique have, however, failed to show any significant difference between untreated skin and skin previously treated with colloidal sulfur or polythionate (21).

The object of the present investigation was to determine not only the penetrability of the sulfur compounds from the polythionates through the skin barrier of the rat but also whether there is a relation between length of time of contact and percentage absorption of S^{35}.

EXPERIMENTAL

The methods of the preparation of labeled polythionates and for the study of the absorption of the sulfur compounds derived from the polythionates into the intact skin of rats have been described elsewhere (22).

According to this method an acid polythionate solution is prepared which contains unlabeled penta- and tetrathionate plus tetrathionate labeled at all 4 sulfur atoms. This solution which is quite stable (26, 27) was stored at room temperature for 2 months before use. During this time the labeled sulfur atoms of the tetrathionate anions were exchanged with the sulfur atoms of the pentathionate anions so that both polythionates were labeled at the time the penetration study was undertaken. A preliminary report of this exchange process appears in THIS JOURNAL (28).

The penetrability of the sulfur compounds from the polythionates was determined by the topical application of the S^{35}-labeled polythionate solution on the clipped area of the back of rats. The experimental animals were divided into three groups of ten rats each. One group was sacrificed one hour after the application of radioactive polythionate, the

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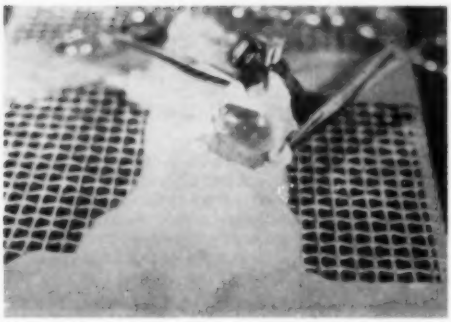

Fig. 1.—View of Selye's air bubble when the overlaying skin has been peeled back without piercing the bubble.

second group four hours later, and the third group twenty-four hours after application. In all groups the area selected for treatment was marked off and the radioactive polythionate solution was applied with a cotton swab. In order to be able to localize the treated skin, a small amount of ink was added to the polythionate solution. The amount of solution applied to the skin varied from 10–30λ per sq. cm.

After the appropriate time interval, the animals were sacrified and the treated skin was cut free from the carcass. Two samples of skin were then obtained from each animal by means of a $^{5}/_{8}$-inch punch (21.5 mm. diameter). One piece of skin was assayed directly for S^{35} by the method of Fingl, *et al.* (19). The companion piece of skin was carefully washed until a survey with a Geiger counter either indicated no activity or a constant count after successive washings and then assayed for S^{35}.

In order to determine the per cent recovery of topically applied S^{35}, the carcasses of four rats in each group were dissolved in a hot solution of sodium hydroxide. The liver of each animal was dissolved and ashed separately. Aliquots of the resulting solutions were assayed for S^{35} by the method of Fingl, *et al.* (19). All observed counts were corrected for background, radioactive decay, and self-absorption (24, 25).

In another series of experiments the radioactive polythionate solution was appied to the skin of the rat in a somewhat different manner. Two small bottle caps, in which $^{1}/_{8}$-inch holes had been drilled in the center, were glued to adjacent areas of the shaven back of rats. The bottle caps had an inside diameter of 2.15 cm. corresponding to a free area of approximately 3 sq. cm. By pipetting 0.8 cc. of radioactive polythionate solution into these small cups and then sealing the small hole with a polyethylene disk, evaporation of the solution was prevented. In this way the area of skin under the cup was in direct contact with the radioactive polythionate solution throughout the entire experiment.

After the appropriate time interval, the animals were sacrified and the radioactive polythionate solution sucked off. The cups were then removed and the treated skin blotted dry with an absorbent tissue. The areas directly under the cups were punched out and treated as described above.

In one experiment the cup technique was used with rats in which the so-called "pouch" of Seyle (Fig. 1) had been produced (23). The pouches were created by injecting a given amount of air under the loose skin of the back of the rat. Two cups (Fig. 2) were then glued to the skin area comprising the pouch of each of 10 animals. Radioactive polythionate was pipetted into these small cups and allowed to remain in contact with the skin of the pouch for twenty-four hours. In this experiment the skin directly under the cup, as well as the skin forming the remainder of the pouch, was removed and assayed.

In a similar experiment skin sections were cut radially from the center of the cup to a few centimeters past the circumference of the cup. These skin samples were fixed in pure methanol for twenty-four hours and then passed through isopropanol and xylene and finally embedded in paraffin. A radioautograph was made by pressing this section against a No Screen X-Ray Film for three months.

RESULTS AND DISCUSSION

The results of the first experiment are summarized in Table I. These data clearly show that S^{35}-labeled compounds derived from the polythionates are rapidly absorbed through the intact skin of the rat after simple swabbing with this material. The data also show that with increasing time of application the amount of S^{35} in the treated skin does not increase even though the total amount of S^{35} (sum of S^{35} in and on the skin sample) decreases. These results, therefore, indicate that the absorbed S^{35} reaches the blood stream and enters the general systemic circulation.

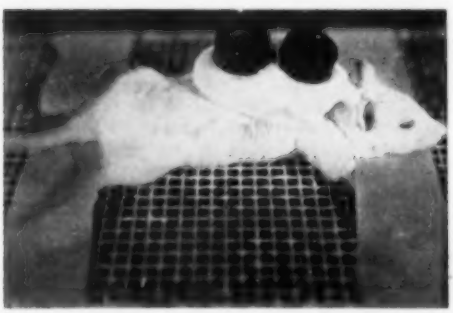

Fig. 2.—Containers for radioactive polythionate solution (bottle caps) on top of Selye's air bubble.

TABLE I.—AMOUNT OF S^{35} IN SKIN FROM ANIMALS SWABBED WITH S^{35}-LABELED POLYTHIONATES[a]

Length of Time of Application, Hr.	Applied S^{35}, %		
	In the Skin Sample[b]	On the Skin Sample[c]	Unaccounted for
1	9.5	51.5	39.0
4	6.0	50.0	44.0
24	6.0	39.0	55.0

[a] Results are averages of 10 animals.
[b] The expression "in the skin" is meant literally and embraces all insoluble sulfur compounds which have been absorbed by the skin, e. g., both the dead and living tissues.
[c] Sulfur compounds which have been removed by washing.

Analyses of liver samples have shown that 33% of all absorbed S^{35} was located in the liver after twenty-

Fig. 3.—Autoradiograph of rat skin exposed to S^{35}-labeled polythionate solution. Enlarged 3 times (linear). The heavy dark area corresponds to the skin which was directly exposed to the radioactive polythionate solution.

four hours of application on the skin. Even one hour after application of S^{35} polythionates 1 per cent of the applied S^{35} could be acounted for in the liver; whereas, 8% was found in the entire carcass.

The findings of the experiment employing the cup technique are tabulated in Table II. The

TABLE II.—AMOUNT OF S^{35} IN SKIN FROM ANIMALS TREATED WITH S^{35} POLYTHIONATES EMPLOYING THE CUP TECHNIQUE[a]

Length of time of Application, Hr.	Applied S^{35}, %			
	In the Skin Sample[b]	On the Skin Sample[c]	Recovered from Cup	Unaccounted for
1	0.5	4.2	58.8	36.5
4	0.9	1.6	55.6	41.9
24	1.3	5.2	52.4	41.1

[a] Results are average of 10 animals.
[b] The expression "in the skin" is meant literally and embraces all insoluble sulfur compounds which have been absorbed by the skin, e.g., both the dead and the living tissue.
[c] Sulfur compounds which have been removed by washing.

results of the cup experiment are qualitatively the same as those of the swab experiments. The per cent of the applied S^{35} found in the skin sample in the cup experiment appears to be considerably less than that found in the swab experiment. This is due to the much larger amount of radioactive polythionate which could be applied to the skin by the cup method as compared to that which could be applied by the swab method. However, the absolute amounts of S^{35} present in the skin samples, when employing either technique, were not significantly different.

In Table III are given the data obtained from the cup experiment in which rats with air bubbles under the skin of their backs were used.

TABLE III.—PER CENT RECOVERY OF S^{35} AFTER A 24-HOUR TREATMENT WITH S^{35}-LABELED POLYTHIONATES EMPLOYING THE CUP TECHNIQUE[a]

Applied S^{35}, %					
Recovered from Cup	In Carcass minus Liver	In Liver	In and on Skin under Cup	In the Remainder of Skin of Bubble	Total Recovery of S^{35}, %
53	27	13	2.5	1	97.5

[a] Results are averages of 10 animals.

The distribution of the applied S^{35}, after an interval of twenty-four hours, is recorded in this table, together with an estimation of the total percentage recovery of S^{35}. It will be noted that the concentration of S^{35} in the liver is comparatively high. In this experiment the unabsorbed S^{35}-labeled polythionates could be recovered so that 97.5% of the applied S^{35} could be accounted for. It will further be noted that 1% of the applied S^{35} was found in the skin of the bubble surrounding the cup.

The autoradiographs obtained and presented in Fig. 3 show a heavy concentration of S^{35} directly under the cup with decreasing amounts of S^{35} as the distance from the center of the cup increases. The time of contact between polythionate solution and skin was 1.5 hours.

The S^{35}-labeled compounds in the skin section apparently were not soluble in isopropanol and xylene, while they appeared to be water-soluble. A corresponding autoradiograph made from a tissue slice previously washed in running water for five minutes showed only traces of radioactivity.

SUMMARY

1. A study has been made of the absorption of S^{35}-labeled compounds derived from topically applied radioactive polythionates by the skin of the adult rat.

2. The amount of S^{35} absorbed by the skin increased with the time of application. Some of the absorbed S^{35} entered the systemic circulation.

3. The data indicate that the degree of absorption of sulfur compounds from topically applied polythionates is independent of the amount of polythionates applied on the skin over the concentration range studied.

4. The uptake of S^{35} into the skin of the rat was also studied by means of autoradiography.

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The Exchange of Sulfur Atoms between Tetrathionate and Pentathionate Anions*

By TORBEN E. NEESBY

The exchange of sulfur atoms between tetrathionate and pentathionate anions has been shown to take place when the alkali salts were present together in a stable diluted aqueous solution. The mixture of tetrathionate and pentathionate salt was partly separated by fractionation after conversion into some new lipophilic organic ammonium polythionates which were only slightly soluble in water.

IN CONNECTION WITH the studies undertaken for the purpose of investigating the absorption of sulfur compounds through the skin from externally deposited polythionates as reported in THIS JOURNAL (1), the question arose whether the sulfur atoms in one species of polythionates, for example tetrathionate, would exchange with the sulfur atoms of pentathionate when these anions were present together in aqueous solution.

The following preliminary study was made to investigate this proposition. Sodium tetrathionate with tagged sulfur atoms in all 4 positions was prepared by the method previously published in THIS JOURNAL (2).

This sodium tetrathionate was dissolved in water and a solution of standard composition of untagged tetrathionate and pentathionate was added.[1] The pH was adjusted to approximately 1 and a trace of cupriacetate was added to assure a stable solution (3). The solution at this time contained 5% polythionates. It was stored for 2 months at ordinary temperature. The problem was then to separate the pentathionate from the tetrathionate to see if the pentathionate had become radioactive. A separation has hitherto been difficult. It has been done by crystallization and handpicking the crystals of different forms (4).

Chemically, the two acids are very much alike but when decomposed by alkali, they render different products (5). Alkali decomposition of tetrathionate yields thiosulfate and sulfite, whereas alkali decomposition of pentathionate gives rise to free elementary sulfur and thiosulfate. This method, however, destroys the polythionates. It has recently been discovered that high molecular organic quaternary ammonium cations will form insoluble complexes with polythionate anions. Employing this finding a fractionation of the polythionates may be accomplished by stepwise precipitation and subse-

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[1] A 5% acid solution of penta- and tetrathionate, similar composition sold under name Dermasulf®.

quent extraction with ethylene dichloride. By using this type of fractionation, the higher polythionates could be separated from the lower, since the higher polythionate complexes are more soluble in ethylene dichloride than the lower polythionate complexes. The quaternary ammonium halide was stirred into an aqueous solution of the polythionates in amounts which corresponded to only a fraction of the stoichiometrically necessary amount for a total precipitation of the polythionates.

$$2RN(CH_3)_3x^- + S_5O_6^{--} \rightarrow (RN(CH_3)_3)_2S_5O_6^{--} + 2X^-$$

and when purified give soft crystals. In less pure state, the organic ammonium polythionates are semicrystalline or waxy substances more or less insoluble in water.

These compounds may be used to indicate the existence of the higher polythionates, e. g., polythionates with more than 6 sulfur atoms.

In a previously concentrated and again diluted polythionate solution a very high molecular weight fraction of polythionates could be precipitated by addition of about 2–5% of the stoichiometrically necessary amount of quaternary ammonium halide. These polythionates

TABLE I

Disk No.	Radioactive Polythionates: Weight of Ppt., Gm.	Radioactive Polythionates: Count per 10 Min.[a]	Nonlabeled Polythionates: Weight of Ppt., Gm.	Nonlabeled Polythionates: Calculated Weight (Gm.) of Pentathionate	Calculated Weight (Gm.) of Tetrathionate	Calculated Weight (Gm.) of Quat. Cation
1	0.2784	2936	0.2759	0.061 (9)	0.033 (2)	0.180 (8)
2	0.2951	2997	0.2884	0.047 (4)	0.049 (1)	0.191 (9)
3	0.2962	3220	0.2959	0.039 (7)	0.059 (7)	0.195 (5)
4	0.3178	3350	0.3090	0.044 (3)	0.060 (1)	0.204 (6)
5	0.2847	3242	0.3047	0.041 (5)	0.061 (2)	0.202 (0)
6	0.3003	3352	0.2997	0.036 (3)	0.064 (4)	0.199 (0)
7	0.3040	3284	0.3025	0.028 (1)	0.073 (0)	0.201 (5)
8	0.3017	3289	0.2912	0.029 (2)	0.068 (0)	0.194 (0)
9	0.3193	3453	0.2984	0.027 (0)	0.072 (5)	0.198 (9)
10	0.2835	3291	0.2864	0.015 (6)	0.078 (8)	0.192 (0)
11	0.3112	3266	0.2949	0.010 (4)	0.086 (5)	0.197 (5)
12	0.2724	3081	0.2837	0.008 (7)	0.093 (5)	0.191 (5)
13	0.0337	1223	0.0427	0.000 (8)	0.008 (9)	0.033 (0)
14	0.0224	825	0.0290	0.000	0.009 (3)	0.019 (7)
15	0.0173	563	0.0199	0.000	0.006 (4)	0.013 (5)
16	0.0144	424				
17	0.0140	369				
18	0.0133	325				

[a] Not corrected for self-absorption but for background—65 counts/10 min.

The following organic ammonium compounds have been tried:

Lauryltrimethyl ammonium chloride (Arquad® 12 Armour).

Cetyldimethyl benzyl ammonium chloride (Winthrop Stearns).

Octadecyl trimethyl ammonium halide (Arquad 18 Armour).

N-Lauryl pyridinium chloride (Hooker).

Dodecyl acetamido dimethyl benzylammonium chloride.

p-tert. Octyl tolyl methyl trimethyl ammonium chloride (Rohm & Haas).

p-Di-isobutyl phenoxyethoxyethyl dimethyl benzyl ammonium chloride (Hyamine,® Rohm & Haas).

N-lauryl-N-methyl morpholinium chloride.

N-lauroyl colamino formyl methyl pyridinium chloride (Emulsol Chem.).

These compounds were all found to give insoluble polythionate complexes. They are, when prepared stoichiometrically, salt-like substances possessed an average sulfur content of 10 to 11 per molecule, as estimated by the method of Kurtenacker (6). When the ethylene chloride solution was evaporated, the residue, which is essentially organic quaternary ammonium polythionates, would decompose in a few days into sulfur crystals and penta- and tetrathionate.

Reports have been published earlier of such high sulfur contents of polythionates (7, 8).

When further precipitations and extractions of the same solution were undertaken, polythionates with lower average number of sulfur atoms were obtained.

Last would come penta- and tetrathionate. The relative amount of these two would vary from a high value in the beginning to a low at the end of the fractionation.

A series of extractions were made of the tagged and 2 month old polythionates mixture, the composition of which is described under Experimental. Another series was made of an almost identically composed untagged polythionate

mixture and the fractions from this solution were analyzed with the Kurtenacker methods. The values of the two solutions are tabulated (Table I).

EXPERIMENTAL

The radioactive polythionate solution was made as follows: 100 cc. of an acid polythionate solution (pH 1.0) containing

Pentationate (as anion)...	2.71 Gm./100 cc.
Tetrathionate (as anion)..	2.24 Gm./100 cc.
Total.....	4.95 Gm./100 cc.

was mixed with 100 cc. of a solution of 4.92 Gm. tetrathionate[2]/100 cc. (as sodium tetrathionate and acidified with HCl to pH 1.0).

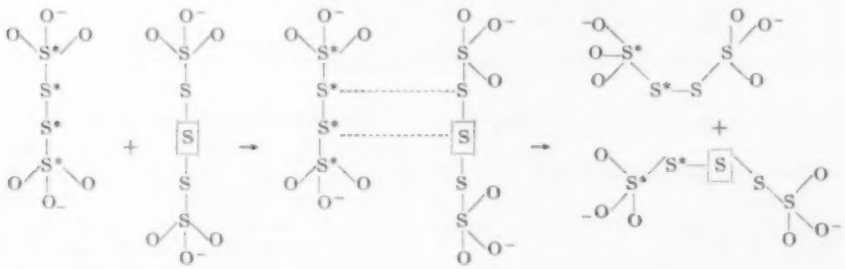
The mixture thus contained:

Pentathionate.....	1.36 Gm./100 cc.
Tetrathionate.....	3.57 Gm./100 cc.
Total.....	4.93 Gm./100 cc.

A similar nonlabeled polythionate was made from barium tetrathionate and barium pentathionate which were dissolved in water and acidified with the calculated amount of dilute sulfuric acid. The $BaSO_4$ was filtered off.

The barium polythionates were prepared according to known methods and recrystallized from water with alcohol until minimum 98% pure (9).

The polythionate solution was partially neutralized with sodium carbonate until the pH was 1.0 and was filled up to 100 cc. with distilled water.



The polythionate solution was analyzed by the Kurtenacker method which gave the results:

Pentathionate (as anion)..	1.43 Gm./100 cc.
Tetrathionate (as anion)..	3.52 Gm./100 cc.
Total.....	4.95 Gm./100 cc.

This was considered a satisfactory close approximation as the titration procedure is not too accurate.

The 50% lauryl trimethyl ammonium chloride in isopropanol was a technical product bought under the name Arquad 12. It was purified by filtration over activated carbon until water-white. The nitrogen was estimated by Kjeldahl and by titration with dioctylsulfosuccinate. The molecular weight according to these methods was 267.5, theoretical 263.9. This corresponds to an equivalent weight of the cation 232.

Extraction of Polythionates from Aqueous Solution with Lauryl Trimethyl Ammonium Chloride and Ethylenedichloride.—Twenty-five cubic centimeters of a 5% radioactive polythionate solution, 0.5 cc. of approximately 50% lauryltrimethylammonium chloride solution in isopropanol, and 3 cc. ethylene dichloride were pipetted into a separatory funnel. The separatory funnel was stoppered and shaken 128 times which took 1½ min.

The emulsion was allowed to break and when the lower layer of ethylene dichloride was sharply separated from the aqueous phase, the ethylene dichloride was drawn off as sharp as possible into a weighed cuplike disk of metal, 1 inch diam. The disk was put into a hood for evaporation.

This procedure was repeated 18 times. After thorough drying at room temperature, first in a hood and then in a desiccator, the disks were weighed and the radioactivity counted.

The experiment was duplicated by using the similarly composed nonlabeled polythionate mixture and the contents of the disks weighed, dried, dissolved in alcohol, and estimated according to Kurtenacker. No starch was needed as indicator. The quaternary ammonium compound forms a yellow precipitate with surplus iodine and this indicates the end point.

DISCUSSION

The table shows that the pentathionate content of the extractions decreases uniformly until it reaches a very low value, while the amount of tetrathionate increases rapidly through the first 3 extractions, then it increases at a slower rate until the abrupt fall indicates the exhaustion of polythionates from the aqueous phase. The tetrathionate concentration in the first extractions is only from ⅓ to ½ of what it is in the later extractions. Nevertheless, the radioactivity of the residue is practically constant throughout the first twelve extractions.

If only the tetrathionate had been radioactive, considerably less radioactivity of the first extractions should have been found.

The self-absorption of the beta radiation by the residues on the disks would be essentially the same, since the weight of each of the first twelve residues is approximately the same.

The radioactivity of the fractions follow the weight of the quaternary ammonium polythionate quite

[2] Indicates the radioactivity.

closely. This can only be considered as indicative of an exchange process of the sulfur atoms between the two polythionate species tetra- and pentathionates.

The presence of cupric ions (3, 4) has insured a stable aqueous solution of the above described composition of pentathionate and tetrathionate at pH 1. This has been verified by the absence of sulfite and thiosulfite into solution.

An exchange of sulfur atoms should, therefore, be possible by an ionic fission according to Foss (10) and Christiansen, *et al.* (11). The exchange could take place, for example, by thiosulfate units according to Eq. 1.

It can be seen that according to this assumption the middle sulfur atom in pentathionate, which is so marked by a frame, cannot be exchanged and will remain untagged. The pentathionate should, therefore, never be able to become more than $^4/_5$ as active as tetrathionate when calculated per atom of sulfur.

Although the first two extractions show a lower radioactivity than the rest, which is in conformity with the postulate, the exactitude is hardly sufficient to consider the finding as proof of the postulate. The relative variation in the counting of activity in this experiment was estimated to be approximately 10%.

The water insolubility of the organic quaternary ammonium polythionate varies with the molecular weight of the organic quaternary ammonium cation. Preliminary investigations on quaternary ammonium compounds of different mole weights have shown that the limit for formation of insoluble polythionate coincides with the limit for the formation of micelles of the quaternary cation.

The solubility of the complex will, however, also vary with the size of the polythionate anion. Thus the solubility varies as follows: (*a*) The lower molecular weight of cation, the higher the solubility; (*b*) the lower molecular weight of polythionate anion, the higher the solubility.

The formation of the insoluble compound is therefore presumably not only due to the electrostatic forces between the ammonium cation and the sulfonium anion but additional intermolecular forces are active.

The shift in color of, for example, bromophenol blue by the addition of a high molecular quaternary ammonium compound might be due to changes in π electron orbits caused by the intermolecular forces. When a series of decreasing molecular weight quaternary ammonium compounds were investigated in respect to their ability to shift the color of a bromophenol blue solution (0.0004 *M*) buffered at pH 3 from yellow to blue, it could be demonstrated that the limit of molecular size was around 8 carbon atoms for the alkyl chain in a trimethyl alkyl ammonium halide.

The insolubility of the high molecular organic quaternary ammonium polythionates could be used to demonstrate the fission of the polythionate anions into thiosulfate units by the following procedure:

A 2% solution of bis(lauryl trimethyl ammonium) penta- and tetrathionate in ethanol was diluted with equal parts of water, whereby a rather stable milky-white suspension would form. When to this suspension, sodium sulfite or sodium carbonate was added the milky suspension would clear up and become transparent. This would go fast with sodium sulfite and slower with other alkaline media. The polythionate anions would, by Foss' exchange-fission, be converted into thiosulfate anions and trithionate anions.

The organic quaternary ammonium thiosulfates and trithionates are readily soluble in water; thus the suspension would become clear.

SUMMARY

1. It has been shown by fractionated precipitation of organic quaternary ammonium polythionates that the sulfur atoms of the two polythionates, pentathionate and tetrathionate, will exchange when the two polythionates are stored together at ordinary room temperature in a diluted stable aqueous solution.

2. It is suggested that the exchange takes place by thiosulfate units in harmony with the reaction mechanisms of polythionates as proposed by Foss.

3. The formation of water-insoluble complexes consisting of high molecular weight organic ammonium cations and polythionate anions are reported as new compounds.

4. The fractionation of polythionates by this method is due to the greater lipophilicity of the longer sulfur chains of the higher polythionates *versus* the hydrophilicity of the sulfonic groups which in the shorter sulfur chain lengths of the lower polythionate result in a greater water solubility.

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Book Notices

Pharmacology and Oral Therapeutics. 11th ed. By EDWARD C. DOBBS. The C. V. Mosby Company, St. Louis, 1956. 579 pp. 14 x 22 cm. Price $9.

This book is designed primarily as a teaching aid for dental students and as a reference book of newer drugs and preparations for the practicing dentist. Most of the text (469 pages) is devoted to pharmacology. The second part (86 pages) is devoted to therapeutics, with a chapter on prescription writing, and with prescriptions for local and systemic medication for the treatment of various conditions. An index is appended. The book should be available to pharmacists who wish to consider detailing the dentist to interest him in writing prescriptions for his patients.

Pharmacognosy. 3rd ed. By EDWARD P. CLAUS. Lea and Febiger, Philadelphia, Pa., 1956. 731 pp. 18 x 26 cm. Price $12.50.

This third edition of the Gathercoal and Wirth textbook has been rearranged by E. P. Claus according to the chemical classification of plant and animal drugs. The monograph form of presentation has been retained with the addition in some monographs of proprietary prescription products containing the drugs. Four new chapters on antibiotics, immunizing biologicals, allergens and allergenic preparations, and pesticides have been added. Appendixes include: A Taxonomic List of Important Drugs; Powdered Drugs (preparation, examination and key for identification); and Cultivation of Drug Plants. A general index is included. The printing is excellent, with sharp cuts and pictures illustrating the text. The book is recommended to teachers of pharmacognosy and should be in all libraries.

Lange's Handbook of Chemistry. 9th ed. Edited by NORBERT ADOLPH LANGE and GORDON M. FORKER. Handbook Publishers, Inc., Sandusky, Ohio, 1956. xvi + 1969 pp. 14 x 20.5 cm. Price $8.50.

The ninth edition of this handbook continues the excellent selective compilation of material that is useful in many fields, but of particular interest to chemists. The eighth edition was reviewed in THIS JOURNAL, **42**, 62(1953). Revision of such a comprehensive reference volume is a prodigious task. New tables have been added and old material has been brought up-to-date. One example of the latter is the change of formula weights in accordance with the latest International Atomic Weight values and recalculation of analytical factors. This book is one that should be available to all chemists, and pharmacists who maintain a reference library will find it a useful addition. The format and printing are exceptionally good for a book that includes 1969 pages between its sturdy but flexible covers. Those who have used Lange's Handbook before will welcome the new edition.

BOOKS RECEIVED

Semimicro Qualitative Organic Analysis: The Systematic Identification of Organic Compounds. 2nd ed. By NICHOLAS D. CHERONIS and JOHN B. ENTRIKIN. Interscience Publishers, Inc., New York, 1957. xiv + 774 pp. 16 x 24 cm. Price $9.

Year Book of Drug Therapy (1956–1597 Series). Edited by HARRY BECKMAN. The Year Book Publishers, Inc., Chicago, 1957. 514 pp. 13 x 20 cm. Price $6.75.

Accepted Dental Remedies 1957. 22nd ed. American Dental Association, Chicago, 1957. xvi + 212 pp. 16 x 20.5 cm. Price $2.

Fusion Methods in Chemical Microscopy. By WALTER C. MCCRONE, JR. Interscience Publishers, Inc., New York, 1957. vii + 307 pp. 15.5 x 23.5 cm. Price $6.75.

Accidental Poisoning in Childhood. By EDWARD PRESS. American Academy of Pediatrics, Evanston, Illinois, 1956. xiv + 131 pp. 14 x 22 cm.

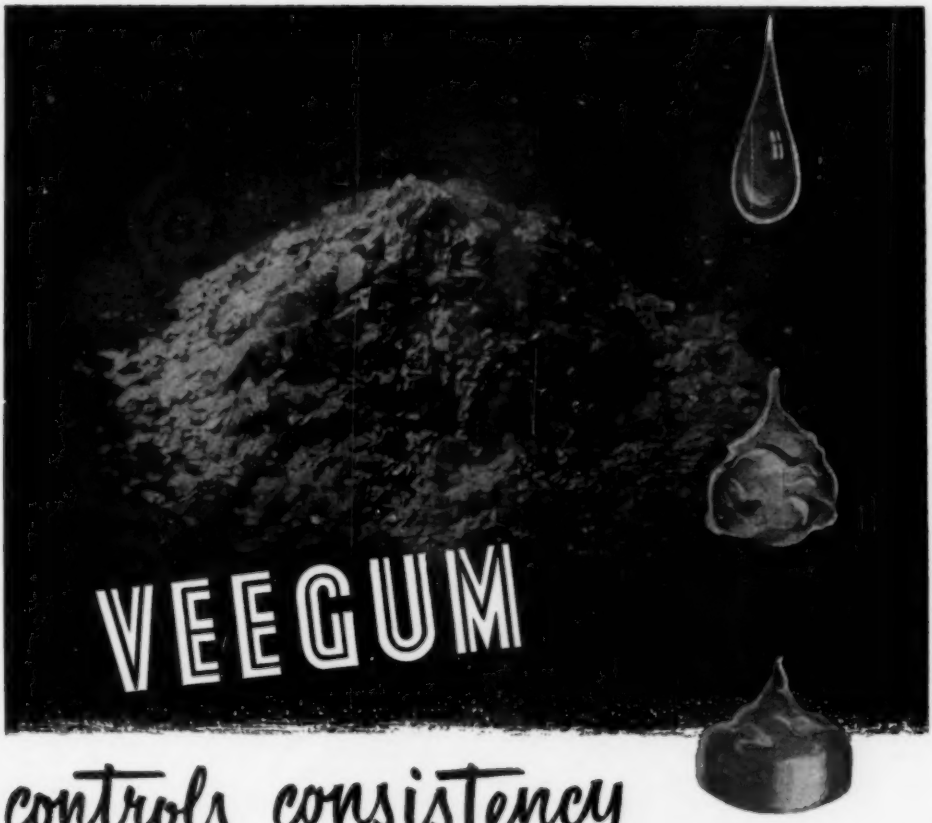
Biochemical Problems of Lipids. Edited by G. POPJÁK and E. LE BRETON. Interscience Publishers, Inc., New York, 1956. xvi + 505 pp. 16 x 25.5 cm. Price $10.75.

The Encyclopedia of Chemistry. Edited by GEORGE L. CLARK and GESSNER G. HAWLEY. Reinhold Publishing Corporation, New York, 1957. xvi + 1,037 pp. 18 x 26 cm. Price $19.50.

Molecules and Crystals in Inorganic Chemistry. 2nd ed. By A. E. VAN ARKEL. Interscience Publishers Inc., New York, 1956. ix + 270 pp. 14.5 x 22.5 cm.

Microcalorimétrie: Applications Physioco-chimiques et Biologiques. By E. CALVET and H. PRAT. Masson et Cie, Paris, 1956. viii + 396 pp. 17 x 25 cm. Price 4.500 fr., paper back. Bound 5.200 fr.

Practical Dermatology. By SAMUEL M. PECK and LAURENCE L. PALITZ. McGraw-Hill Book Company, Inc., New York, 1956. xvii + 380 pp. 14 x 21 cm. Price $7.



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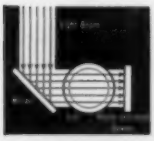

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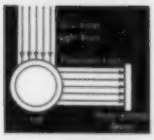

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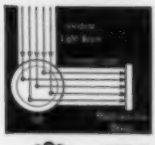

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